10568436

PI 1142211

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

March 09, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/377,733*
FILING DATE: *May 03, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/13765*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. LAWRENCE
Certifying Officer

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

05-06-02

60277733.050302

PTO/SB/16 (10-01)

Approved for use through 10/31/2002. OMB 0651-0032

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL770157485US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Brian	Agnew	Eugene, Oregon
Kyle	Gee	Springfield, Oregon
Richard	Haugland	Eugene, Oregon

☑ Additional inventors are being named on the 1 separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Novel Bapta-Based Chelator Compositions and Methods for Detection and Isolation of Phosphophorylated Biomolecules

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☑ Customer Number 23358 → Type Customer Number here

OR

Customer Number Bar Code Label here 23358

PATENT TRADEMARK OFFICE

☐ Firm or Individual Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification	Number of Pages 117	☐ CD(s), Number	
☑ Drawing(s)	Number of Sheets 6	☐ Other (specify)	
☐ Application Data Sheet. See 37 CFR 1.76			

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

		FILING FEE AMOUNT ($)
☑	Applicant claims small entity status. See 37 CFR 1.27.	
☐	A check or money order is enclosed to cover the filing fees	
☑	The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 13-3900	$80.00
☐	Payment by credit card. Form PTO-2038 is attached.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☑ Yes, the name of the U.S. Government agency and the Government contract number are: National Cancer Institute 1 R33 CA093292-01

Respectfully submitted,

SIGNATURE Koren J. Anderson

Date 5/3.02

TYPED or PRINTED NAME Koren J. Anderson, Ph.D.

REGISTRATION NO. (if appropriate) 51,061

Docket Number:

TELEPHONE (541) 984-5656

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (02-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number	

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Jixiang	Liu	Eugene, Oregon
Vladimir	Martin	Eugne, Oregon
Wayne	Patton	Eugene, Oregon
Thomas	Steinberg	Eugene, Oregon

Number 1 of 1

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

PROVISIONAL PATENT APPLICATION

FOR

UNITED STATES UTILITY PATENT

TITLE: **NOVEL BAPTA – BASED CHELATOR COMPOSITIONS AND METHODS FOR DETECTION AND ISOLATION OF PHOSPHOPHORYLATED BIOMOLECULES**

INVENTORS: **BRIAN AGNEW;**
KYLE GEE;
RICHARD HAUGLAND;
JIXIANG LIU;
VLADIMIR MARTIN;
WAYNE PATTON; AND,
TOM STEINBERG.

NOVEL BAPTA COMPOSITIONS AND METHODS FOR STAINING PHOSPHOPHORYLATED BIOMOLECULES

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made in part with government support under grant number 1 R33 CA093292-01, awarded by the National Cancer Institute. The United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to novel 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA) compositions and methods for use in the staining of phosphorylated biomolecules.

BACKGROUND OF THE INVENTION

Phosphorylation is a process by which a phosphate group is added or removed from a biomolecule, typically a protein. The process of reversible phosphorylation is a key feature of cellular regulation, including, signal transduction, gene expression, cell cycle regulation, cytoskeletal regulation and apoptosis. See, e.g., PROTEIN PHOSPHORYLATION (Marks F. ed., 1996); Hunter, "Signaling – 2000 and beyond," *Cell 100*:113-127 (2000). Principally two enzymes (kinases and phosphatases) modulate reversible protein phosphorylation, which add phosphate groups and which remove phosphate groups, respectively, from molecules. Phosphorylation is a key feature of protein production, and thus phosphorylated proteins must be able to be identified if the proteome is to be fully understood, however to date no practical methods exist for the systematic parallel analysis of the phosphorylation status of large sets of proteins involved in the regulatory circuitry of cells and tissues. See Wilkins *et al, Genetic Eng. Rev. 13*:19 (1995).

Signal transduction is an example of protein phosphorylation that is critical for cellular regulation. After an extracellular stimulatory factor binds to its recognized cell surface receptor, signal transduction is initiated, often by a specific cellular protein kinase. Theses kinases subsequently phosphorylate the target molecule resulting in an altered activity and a continued cellular response to the signal. *See, e.g.*, Nishizuka, "Studies and perspectives of protein kinase C," *Science 233*:305-312 (1986). With the discovery that many oncogenes encode protein kinases, it has become ever more important to researchers studying cancer to

develop techniques to analyze the relative states of phosphorylation of a molecule involved in signal activation before and after the signal is initiated in a cell. Moreover, it is not enough for researchers to simply identify whether a protein is a phosphorylated protein or not, it is has become additionally essential for researchers to identify the site of phosphorylation on proteins. The phosphorylation of proteins on serine, threonine and tyrosine amino acid residues are the most common sites of phosphorylation in eukaryotic cells. *See, e.g.*, Guy *et al.* "Analysis of Cellular Phosphoproteins by Two-Dimensional Gel Electrophoresis: Applications for Cell Signaling in Normal and Cancer Cells," *Electrophoresis 15*:417-440 (1994). Thus, the focus for researchers in understanding protein phosphorylation occurs at two levels. The first level of analysis requires a determination of *whether* a protein is a phosphoprotein, and the second level of analysis requires the identification of *where* the amino acid or amino acids at which phosphorylation occurs. The present invention provides materials and methods for both levels of analysis.

Currently, phosphoproteins are most often detected by autoradiography after incorporation of ^{32}P or ^{33}P into cultured cells or after incorporation into subcellular fractions by protein kinases *See, e.g.*, Yan et al., "Protein Phosphorylation: Technologies for the Identification of Phosphoamino Acids," *J. Chromatogr. A. 808*:23-41 (1998); Guy, G., Phillip, R. and Tan, Y. Electrophoresis 15 (1994) 41798. Such approaches are restricted to a limited range of biological materials, such as tissue culture samples and analysis of clinical samples would require *in vivo* labeling of patients, which is not feasible. Several alternatives to radiolabeling have also been developed over the years.

Phosphoproteins can also be detected by immunoblotting and immunoprecipitation. *See, e.g.*, Soskic *et al.*, "Functional Proteomics Analysis of Signal Transduction Pathways of the Platelet-Derived Growth Factor Beta Receptor," *Biochemistry 38*:1757-1764 (1999); Watty *et al.*, "The In Vitro and In Vivo Phosphotyrosine Map of Activated MuSK," *Proc. Nat'l. Acad. Sci. USA. 97*:4585-4590 (2000). Though high quality antibodies to phosphotyrosine are commercially available, antibodies that specifically recognize phosphoserine and phosphothreonine residues have been more problematic, often being sensitive to amino acid sequence context; the reliability of these antibodies has been questioned because of potential steric hindrances between the interaction of these antibodies and the phosphoproteins. Moreover, when phosphoproteins are not enriched prior to detection with the antibody, the presence of unrelated proteins co-migrating with the protein of interest may lead to false

positive signals. Immunoblotting is best performed after blocking unoccupied sites on the solid-phase support with protein solutions, which interferes with microchemical analysis. Removal of the antibody and stain require relatively harsh treatments (i.e. heating to 65°C, incubation with 0.1% SDS and 1 mM DTT). This also poses problems with subsequent use of the protein for sequencing and mass spectrometry. For immunoprecipitation only the anti-phosphotyrosine antibodies display binding that is tight enough to allow effective immunoprecipitation. Therefore, identification of phosphorylated proteins using immunoblotting and immunoprecipitation techniques is limited to proteins containing phosphorylated tyrosine residues. *See* McLachlin & Chait, *supra.*

Alternatively, phosphorylated proteins can be identified using chromogenic dyes, but with limited success. The cationic carbocyanine dye "Stains-All" (1-ethyl-2-[3-(3-ethylnaphtho[1,2d]thiazolin-2-ylidene)-2-methylpropenyl]-naphtho[1,2d]thiazolium bromide) stains RNA, DNA, phosphoproteins and calcium-binding proteins blue while unphosphorylated proteins are stained red. Stains-All is not routinely used to detect phosphoproteins due to poor specificity and low sensitivity. Stains-All is at least 10 times less sensitive than Coomassie Brilliant Blue and several orders of magnitude less sensitive than ^{32}P-autoradiography. Another chromogenic method the GelCode Phosphoprotein detection kit (Pierce Chemical Company, Rockford, IL) is designed to detect phosphoproteins in gels, however this method posses many limitations. According to this method phosphoproteins are detected in gels through alkaline hydrolysis of phosphate esters of serine or threonine, precipitation of the released inorganic phosphate with calcium, formation of an insoluble phosphomolybdate complex and then visualization of the complex with a dye such as methyl green, malachite green or rhodamine B (as described in Cutting and Roth (1973)]. The detection sensitivity of the staining method is considerably poorer than Coomassie Blue staining, with 80-160 ng of phosvitin, a protein containing roughly 100 phosphoserine residues, being detectable by the commercialized kit. The staining procedure is fairly complex (involving seven different reagents) and alkaline hydrolysis requires heating gels to 65° centigrade, which causes the gel matrix to hydrolyze and swell considerably. Since phosphotyrosine residues are not hydrolyzed by the alkaline treatment, proteins phosphorylated at this amino acid residue escape detection by the method. *See, e.g.*, Green *et al.*, "Differential Staining of Phosphoproteins on Polyacrylamide Gels with a Cationic Carbocyanine Dye," *Anal. Biochem. 56*: 43-51 (1973); Hegenauer *et al.*, "Staining Acidic

Phosphoproteins (Phosvitin) in Electrophoretic Gels," *Anal. Biochem. 78*:308-311 (1977); Debruyne, "Staining of Alkali-Labile Phosphoproteins and Alkaline Phosphatases on Polyacrylamide Gels," *Anal. Biochem. 133*:110-115 (1983); JAPANESE ACRIDINE PHOSPHOPROTEIN STAIN REFERENCE. Dyes for the phosphate-specific fluorescence labeling in which a BODIPY dye was attached to a reactive imidazole group has been developed for the detection of pepsin phosphorylation. See U.S. Pat. No. 5,512,486; Wang & Giese, "Phosphate-Specific Fluorescence Labeling of Pepsin by BO-IMI," *Anal. Biochem. 230*:329-332 (1995).

In addition to detecting phosphoproteins, two methods for the chemical derivatization and enrichment of phosphopeptides resulting in isolation of phosphopeptides from complex mixtures exist. See, e.g. *Goshe et al.*, "Phosphoprotein isotope-Coded Affinity Tag Approach For Isolating and Quantitating Phosphopeptides in Proteome-Wide Analyses," *Anal. Chem. 73*:2578-2586 (2001). The first method involves oxidation of cysteine residues with performic acid, alkaline hydrolysis to induce β-elimination of phosphate groups from phosphoserine and phosphothreonine residues, addition of ethanedithiol, coupling of the resulting free sulfhydryl residues with biotin, purification of phosphoproteins by avidin affinity chromatography, proteolytic digestion of the eluted phosphoproteins, a second round of avidin purification and then analysis by mass spectrometry (Oda, Y., Nagasu, T., and Chait, B. Nature Biotechnol. 19 (2001) 379). The first method uses beta-elimination to remove phosphate groups that are replaced with a tag, as exemplified with biotinylated thiol groups wherein the peptides could then be isolated by chromatography on avidin resins. An alternative method requires proteolytic digestion of the sample, reduction and alkylation of cysteine residues, N-terminal and C-terminal protection of the peptides, formation of phosphoramidate adducts at phosphorylated residues by carbodimide condensation with cystamine, capture of the phosphopeptides on glass beads coupled to iodoacetate, elution with trifluoroacetic acid and evaluation by mass spectrometry (Zhou *et al.*, "A Systematic Approach to the Analysis of Protein Phosphorylation," *Nat. Biotechnol. 19*:375-378 (2001). These methods are time consuming, require purified phosphopeptides and are limiting in what can be isolated. Both procedures identified the monophosphorylated trypsin peptide fragment from the test protein, but both failed to detect the tetraphosphorylated peptide fragment.

Alternatively a method for combining chemical modification and affinity purification has been shown for the characterization of serine and threonine phosphopeptides in proteins based on the conversion of phosphoserine and phosphothreonine residues to *S*-(2-mercaptoethyl)cysteinyl or β-methyl-*S*-(2-mercaptoethyl)cysteinyl residues by β-elimination/1,2-ethanedithiol addition, followed by reversible biotinylation of the modified proteins. After trypsin digestion, the biotinylated peptides were affinity-isolated and enriched, followed by the subsequent structural characterization by liquid chromatography/tandem mass spectrometry (LC/MS/MS). See Adamczyk *et al.*, "Selective Analysis of Phosphopeptides Within a Protein Mixture by Chemical Modification, Reversible Biotinylation and Mass Spectrometry," *Rapid. Commun. Mass Spectrom. 15*:1481-1488 (2001).

Fluorescence detection methods appear to offer the best solution to global protein quantitation in proteomics. However, currently, there is no satisfactory method for the specific and reversible fluorescent detection of gel-separated phosphoproteins from complex samples. Derivatization and fluorophore labeling of phosphoserine residues by blocking free sulfhydryl groups with iodoacetate or performate, alkaline β-elimination of the phosphate residue, addition of ethanedithiol, and reaction of the resulting free sulfhydryl group with 6-iodoacetamidofluorescein has been demonstrated in capillary electrophoresis using laser-induced fluorescence detection and similar reactions have been performed on protein microsequencing membranes. However, neither method is suitable for detection of phosphoproteins directly in gels. One problem with the approach is that a delicate balance must be struck between the base and the ethanedithiol in order to achieve elimination of the phosphate group from the serine residue and addition of the ethane dithiol to the resulting dehydroalanine residue without hydrolysis of the peptide backbone.

Several instrument-based methods are also available for the determination of protein phosphorylation such as ^{31}P NMR [105, 106], mass spectrometry [*See, e.g.*, Resing & Ahn, "Protein phosphorylation analysis by electrospray ionization-mass spectrometery," *Methods Enzymol. 283*:29-44 (1997); Aebersold & Goodlett, "Mass spectrometry in proteomics," *Chem. Rev. 101*:269-295 (2001). Affolter, M., Watts, J., Krebs, D., and Aebersold, R. Anal. Biochem. 223 (1994) 74; Liao, P., Leykam, J., Andrews, P., Gage, D., and Allison, J. Anal. Biochem. 219 (1994) 9; Oda, Y., Huang, K., Cross, F., Cowburn, D., and Chait, B. Proc.

Natl. Acad. Sci. USA 96 (1999) 6591) and protein sequencing [110]. Mass spectrometry has been used to provide the molecular mass of an intact phosphorylated protein by comparing the mass of the unphosphorylated protein to the phosphorylated protein. *See, e.g.*, McLachlin & Chait, "Analysis of Phosphorylated Proteins and Peptides by Mass Spectrometry," *Current Opin. Chem. Biol. 5*:591-602 (2001). But this is limiting in that researches must have purified amounts of both proteins. While these procedures accurately characterize the phosphorylation status of proteins and peptides, they are unsuitable for high-throughput screening of phosphorylated substrates. The techniques are generally used after a phosphoprotein has been identified by autoradiography or immunoblotting with anti-phosphotyrosine antibody. Though methods have recently been introduced to directly quantify the relative abundance of phosphoproteins in two different samples by mass spectrometry through culturing different cell populations in ^{15}N-enriched and ^{14}N-enriched medium, the linear dynamic range of such methods has explicitly been demonstrated over only a 10-fold range [109]. Ion suppression phenomena associated with mass spectrometry prevents stoichiometric comparison of different phosphoproteins by such techniques.

For analysis of the site(s) of phosphorylation on molecules, a more detailed analysis of the sites of phosphate attachment and stoichiometery often requires the examination of peptide fragments of the phosphoprotein of interest. Such fragments are usually generated by digestion of the phosphoprotein with proteases such as trypsin. However, mass spectrography analysis of proteolytic digests of proteins rarely provides full coverage of the protein sequence, and regions of interests often go undetected. In addition, protein phosphorylation is often sub-stoichiometric, such that the phosphoproteins are present in lower abundance than other peptides from the protein of interest. Therefore, the identification and characterization of phosphoproteins would be improved greatly by highly selective methods of enriching phosphopeptides prior to analysis with mass spectrography.

Currently, selective enrichment of phosphopeptides from complex mixtures is performed using immobilized metal affinity chromatography, known as IMAC. Using this technique, metal ions such as Fe^{3+} are bound to a chelating support prior to the addition of a complex mixture of peptides or proteins. *See, e.g.*, Posewitz & Tempst, "Immobilized Gallium(III) Affinity Chromatography of Phosphopeptides," *Anal. Biochem. 71*:2883-2892 (1999). Phosphopeptides that bind to the column can be released using high pH or phosphate buffer, though the latter step usually requiring a further desalting step before analysis with mass

spectrography. Resins with iminodiacetic acid and nitrilotriacetic acid chelators are known and are available commercially. *See, e.g.*, Neville *et al.*, "Evidence for Phosphorylation of Serine 753 in CFTR Using a Novel Metal-Ion Affinity Resin and Matrix-Assisted Laser Desorption Mass Spectrometry," *Protein Sci. 6*:2436-2445 (1997). However, there are several complications using current techniques, including loss of phosphopeptides that do not bind to the column (low affinity), difficulty in the subsequent elution of phosphorylated peptides, and background from non-phosphorylated peptides that have affinity for immobilized metal ions (low specificity).

Mass spectrometry detection of separated peptides and direct matrix-assisted laser desorption/ionization (MALDI) analysis of phosphopeptides bound to an IMAC support has been demonstrated. *See* Zhou *et al.*, "Detection and Sequencing of Phosphopeptides Affinity Bound to Immobilized Metal Ion Beads by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry," *J. Am. Soc. Mass. Spectrom. 11*:273-283 (2000). IMAC has also been coupled directly to mass spectrometry instruments on-line, or with superseding separation techniques, such as HPLC and capillary electrophoresis (CE), for the detection and analysis of phosphopeptides.

There are a variety of chelating moieties that use poly-carboxylate binding sites to selectively bind monovalent and divalent metal cations, and these are often used in fluorescent calcium ion indicators. Some indicators that are based on the BAPTA (1,2-bis-(2-aminophenoxyethane)-N,N,N',N'-tetracetic acid) chelator have shown high selectivity for calcium ions inside cells. Examples of these indicators are, for example, quin-2, fura-2, indo-1 (U.S. Pat. No. 4,603,209 to Tsien *et al.* (1986)); fluo-3 and rhod-2 (U.S. Pat. No. 5,049,673 to Tsien et al. (1991) and FURA RED™ (Molecular Probes, Inc., Eugene, OR, trademark for 1-[6-amino-2-2-(5-oxo-2-thioxo-4-thiazolidinylidene methyl-5-benzofuranyloxyl]-2-('2'-2-amino-5'-methyl-phenoxy) ethane N, N, N', N'-tetracetic acid and the tetraacetyloxymethyl ester thereof, as described in U.S. Pat. No. 4,849,362 to De Marinis *et al.* (1989)). A family of BAPTA-based indicators that are selective for calcium ions are described in HAUGLAND, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS. Examples of BAPTA-based metal chelators are also described in U.S. Pat. No. 5,773,227 to Kuhn *et al.* (1998); U.S. Pat. No. 5,453,517 to Kuhn *et al.* (1995); U.S. Pat. No. 5,516,911 to London *et al.* (1996); U.S. Pat. No. 5,501,980 to Katerinopoulos *et al.* (1996); and U.S. Pat. No. 5,459,276 to Kuhn *et al.* (1995).

The present invention overcomes the limitations and disadvantages of currently disclosed methods for staining phosphorylated proteins and thus provides a long felt need for a rapid and effective method for detecting and isolating phosphoproteins for further analysis. The present invention can accurately identify phosphoproteins with only one phosphate group and in a simple method that does not require multiple steps or pre-treatment of the sample. Importantly, the present invention is the first method to provide a means for accurately identifying the phosphorylated proteome and allows for the quantitative identification of increased phosphorylation of proteins. In addition, as will be described below, the materials and methods of the present invention are not limited to the detection and/or separation of phosphorylated proteins.

SUMMARY OF THE INVENTION

Methods and compositions are provided for staining phosphorylated biomolecules in a biological sample. The methods involve combining phosphorylated biomolecules with BAPTA compounds, an acid and a gallium salt to form an acidic mixture, wherein trivalent gallium ions bind with high affinity to the BAPTA compounds and phosphorylated biomolecules forming a tertiary complex. The mixture can be formed by the individual addition of each component or in combination, preferably the acid, gallium salt and BAPTA compounds are added together before mixing with the phosphorylated biomolecules. The resulting tertiary complex can be visualized when the BAPTA optionally comprises a detectable label.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A: Ovalbumin stained with Compound 2 in a polyacrylamide gel. Electrophoretogram of a protein mixture run on a gel and stained for phosphoprotein using the labeled BAPTA compound (Compound 2) described in Example 1. The protein mixture was loaded at ca. 500 μg and contained nine total proteins, one of which was a phosphoprotein (ovalbumin) that contains two phosphate groups. The figure demonstrates specific detection of ovalbumin against a background of very low or no staining of eight un-phosphorylated proteins.

Figure 1B: Total proteins stained with SYPRO™ Ruby protein gel stain. Electrophoretogram of the protein mixture stained for phosphoprotein (Figure 1A) and then post-stained with SYPRO™ Ruby protein gel stain for total proteins, as in Example 2. The figure demonstrates detection of all nine proteins, of which only one (ovalbumin) is a phosphoprotein.

Figure 2A: Ovalbumin stained with Compound 1 on a PVDF membrane. Electrophoretogram of a protein mixture blotted to PVDF membrane after SDS gel electrophoresis and stained for phosphoproteins with the labeled BAPTA compound (Compound 1) described in Example 6. The figure demonstrates specific detection of ovalbumin against a background of very low or no staining of 5 un-phosphorylated proteins.

Figure 2B: Total proteins stained with SYPRO™ Ruby blot stain. Electrophoretogram of a protein mixture blotted to PVDF membrane after SDS gel electrophoresis and stained for phosphoproteins with Compound 1 as in Example 6 (Figure 2A) and then post-stained with SYPRO™ Ruby protein blot stain for total proteins, as in Example 2. The trace shown demonstrates detection of all six proteins, of which one (ovalbumin) is a phosphoprotein.

Figure 3: Sensitivity and linear dynamic range of the phosphoprotein gel stain. Fig. 3A: Comparison of 5 phosphoproteins. Proteins were loaded in two-fold dilution series on SDS minigels; each protein sample was done in series in 4 replicate gels. Gels were fixed, stained, and images obtained as in Example 1. The phosphoproteins were α-casein (7 or 8 phosphates); dephosphorylated α-casein (1 or 2 phosphates); β-casein (5 phosphates); ovalbumin (2 phosphates), and pepsin (1 phosphate). Figure 3B: Comparison of pepsin (1 phosphate with bovine serum albumin (BSA) no phosphates). Quantitative data were manually obtained with the Image Gauge Analysis software (Fuji Photo Film. Co. LTD) and graphed in Microsoft Excel.

Figure 4. Detection of protein phosphatase activity
Results are shown for α-casein and pepsin, as done in example 8. The gel was stained for phosphoprotein detection and then for total protein.

Figure 5: Phosphopeptide Precipitation with Compound 5

A mixture of two non-phosphorylated peptides (Angiotensin I and II, MW's 1297 and 1046, respectively) and two phosphorylated peptides, pT/pY and RII (MW's 1670 and 2193, respectively), was combined with Compound 5, incubated for 1 hour and centrifuged for 5 minutes. The resulting supernatants (bottom spectra in panels) and pellet precipitates (top spectra in panels) were analyzed by MALDI mass spectrometry. Panel A shows the non-phosphorylated peptides exclusively in the supernatants, while figures B and C show the two phosphopeptides of greater than 95% purity in the pellets.

Figure 6: Analysis of phosphorylated peptides eluted from a BAPTA gallium ion affinity column.

(A) Differential MALDI analysis of purified β-casein phosphoserine peptides after dephosphorylation (left peaks in pairs) and subsequent derivatization with methylamine (right peaks in pairs). Results show all three peptides are phosphoserine derivatives by methylamine addition. A and B were monophosphorylated (+ 31 amu for methylamine) and C was triphosphorylated (+93 amu for 3 methylamines). (B) MALDI-TOF MS profile of eluted phosphopeptides from BAPTA-agarose column versus commercially available metal affinity columns (Pierce Chemical Co., Inc.). Under the conditions used, the BAPTA-agarose column shows all expected phosphopeptides (arrows) purified from a complex peptide mix. (C) Control peptide (MW=1870) with one phosphothreonine and one phosphotyrosine residue after treatment with strong base (-98 amu) and methylamine. Results show elimination of single phosphate only (-98 amu from threonine) with no subsequent addition of methylamine (+32 amu) confirming a single phosphothreonine residue. Phosphotyrosine is determined by a lack of modification under elimination conditions.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

I. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention is related. The following terms are defined for purposes of the invention as described herein.

The term "activity" as used herein, refers to the function of a molecule in its broadest sense. It generally includes, but is not limited to, biological, biochemical, physical or chemical functions of the molecule. For example, enzymatic activity, ability to interact with other molecules, ability to facilitate, activate, stabilize, inhibit, suppress, or destabilize the function of other molecules, capacity to modify other molecules, capacity to be modified by other molecules, stability, ability to localize to certain subcellular localizations either inside or outside a cell, are all considered to be within the definition of this term as used herein.

The term "affinity" as used herein refers to the strength of the binding interaction of two molecules, such as a metal chelating compound and a metal ion.

The term "alkyl" as used herein refers to a straight, branched, or cyclic hydrocarbon chain containing between one and twenty carbon atoms, typically between about one and about ten carbon atoms (e.g., methyl, ethyl, n-propyl, iso-propyl, cyclopropyl, n-butyl, iso-butyl, tert-butyl, cyclobutyl, adamantly, noradamantyl, and the like) and attached to the compound by a carbon atom. Straight, branched or cyclic hydrocarbon chains having eight or fewer carbon atoms will also be referred to herein as "lower alkyl." The hydrocarbon chains may further include on ore more degrees of unsaturation, i.e., one or more double or triple bonds (e.g., vinyl, propargyl, allyl, 2-buten-1-yl, 2-cyclopenten-1-yl, 1,3,-cyclohexadien-1-yl, 3-cyclohexen-1-yl, 2-cyclopenten-1-yl, 1,3-cyclohexadien-1-yl, 3-cyclohexen-1-yl and the like). Alkyl groups containing double bonds will also be referred to herein as "alkenes" and Alkyl groups containing triple bonds will be referred to herein as "alkynes". However, as used in context with respect to cyclic alkyl groups, the combinations of double and/or triple bonds do not include those bonding arrangements that render the cyclic hydrocarbon chain aromatic. Hydrocarbon chains having one or more noncarbon atoms (i.e. heteroatoms such as N, S, O, P) in the chain will also be referred to herein as heteroalkyl. "Alkyl" further includes one or more substitutions at one or more carbon atoms of the hydrocarbon fragment or radical. Such substitutions include, but are not limited to: aryl; heterocycle; halogen (to form, e.g., trifluoromethyl, --CF_3); nitro (--NO2); cyano (--CN); hydroxyl (also referred to herein as "hydroxy"), alkoxyl (also referred herein as alkoxy) or aryloxyl (also referred to

herein as "aryloxy," –OR); thio or mercapto, alkyl, or arylthio (--SR); amino, alkylamino, arylamino, dialkyl— or diarylamino, or arylalkylamino (--NRR'); aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbony, diarylaminocarbonyl or arylalkylaminocarbonyl (--C(O)NRR'); carboxyl, or alkyl— or aryloxycarbonyl (--C(O)OR); aldehyde; aryl— or alkylcarbonyl (RC(O)--); iminyl, or aryl— or alkyliminyl (--C(==NR)R'); where R and R' independently are hydrogen, aryl or lower alkyl as defined herein. Substituents that include one or more heteroatoms (i.e., heterocycle, heteroaryl, and heteroaralkyl) are defined by analogy to the above-described terms. For example, the term "heterocycleoxy" refers to the group –OR, where R is heterocycle as defined below.

The term "amino" or "amine group" refers to the group –NR'R'', where R' and R'' are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heteroaryl, and substituted heteroaryl. In a primary amino group, both R' and R'' are hydrogen, whereas in a secondary amino group, either, but not both, R' or R'' is hydrogen.

The term "aryl" as used herein refers to cyclic aromatic carbon chains having twenty or fewer carbon atoms, e.g., phyl, naphthyl, biphenyl, and antrhacenyl. One or more carbon atoms of the aryl group may also be substituted with, e.g., alkyl; aryl; heterocycle; halogen; nitro; cyano; hydroxyl, alkoxyl or aryloxyl; thio or mercapto, alkyl-, or arylthio; amino, alkylamino, arylamino, dialkyl-, diaryl-, or arylalkylamino; aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl, or arylalkylaminocarbonyl; carboxyl, or alkyl- or aryloxycarbonyl; aldehyde; aryl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or arylsufonyl; hydroximinyl, or aryl- or alkoximinyl. In addition, two or more alkyl or heteroalkyl substituents of an aryl group may be combined to form fused aryl-alkyl or aryl-heteroalkyl ring systems (e.g., tetrahydronaphthyl). Substituents including heterocyclic groups (e.g., heterocycleoxy, heteroaryloxy, and heteroaralkylthio) are defined by analogy to the above-described terms.

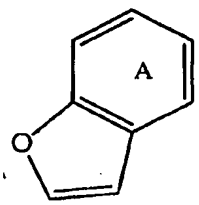
The term "aryloxy" refers to the group aryl-O— or heteroaryl-)—.

The term "arylalkyl" or "aralkyl" as used herein refers to an aryl group that is joined to a parent structure by an alkyl group as described above, e.g., benzyl, α-methylbenzyl, phenethyl, and the like.

The term "aqueous solution" as used herein refers to a solution that is predominantly water and retains the solution characteristics of water. Where the aqueous solution contains solvents in addition to water, water is typically the predominant solvent.

The term "BAPTA" as used herein refers to analogs of the ion-chelating compound (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid) and salts thereof wherein two phenyl rings (ring A and B) are joined by a C_1-C_3 hydrocarbon bridge, where each phenyl ring is optionally substituted by one or more labels and/or other substituents that adjust the ion-binding affinity, solubility, spectral properties or other physical properties of the compound. The term "BAPTA" also refers to a compound that is inherently fluorescent.

The term "benzofuran" or "benzofuran derivative" as used herein refers to a fluorescent dye label generally having the structure below.



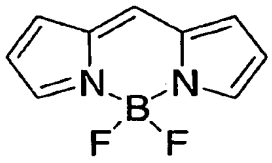
Wherein the benzofuran can be substituted or unsubstituted and can be attached to the BAPTA compound by a single covalent bond at any carbon atom of the BAPTA compound or as a fused ring wherein ring A on the benzofuran would also be ring A or B on the BAPTA

compound. The oxygen atom may be present at either hetoatom position adjacent to the benzene ring. The benzofuran may optionally be substituted or unsubstituted as depicted.

The term "biotin" as used herein refers to any biotin derivative, including without limitation, substituted and unsubstituted biotin, and analogs and derivatives thereof, as well as substituted and unsubstituted derivatives of caproylamidobiotin, biocytin, desthiobiotin, desthiobiocytin, iminobiotin, and biotin sulfone.

The term "biotin-binding protein" as used herein refers to any protein that binds selectively and with high affinity to biotin, including without limitation, substituted or unsubstituted avidin, and analogs and derivatives thereof, as well as substituted and unsubstituted derivatives of streptavidin, ferritin avidin, nitroavidin, nitrostreptavidin, and Neutravidin™ avidin (a de-glycosylated modified avidin having an isoelectric point near neutral).

The term "borapolyazaindacene" or "borapolyazaindacene derivative" as used herein refers to a compound generally having the formula:



Wherein the borapolyazaindacene can be substituted or unsubstituted and is attached a BAPTA compound by a linker or a single covalent bond.

The term "buffer" as used herein refers to a system that acts to minimize the change in acidity or basicity of the solution against addition or depletion of chemical substances.

The term "carbonyl" as used herein refers to the functional group –C(O)--. However, it will be appreciated that this group may be replaced with well-known groups that have similar electronic and/or steric character, such as thiocarbonyl (--C(S)--); sulfinyl (--S(O)--); sulfonyl (SO2)--), phosphonyl (--PO2--).

The term "carboxy" or "carboxyl" refers to the group –R'(COOH) where R' is alkyl substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heterocyclic, heteroaryl, or substituted heteroaryl.

The terms "cell or cells" as used herein refers to an autonomous self-replicating unit composed of protoplasm delimited by a cell membrane that may constitute an organism (in the case of unicellular organisms) or be a subunit of multicellular organisms (in which individual cells may be more or less specialized differentiated) for particular functions. Cell or cells as used in the instant application also includes cells in cell culture medium, single cells, isolated cells, single-cell organisms; and "portions thereof" including, but not limited to, cell extracts, cell homogenates, cell lysates, spinal fluid and subcellular components.

The term "complex" as used herein refers to the association of two or more molecules, usually by non-covalent bonding, e.g., with a metal chelator and a metal ion complexed with (i.e., non-covalently bound to) a protein.

The term "detectable response" as used herein refers to a change in, or occurrence of, a signal that is detectable either by observation or by instrumentation. Typically, the detectable response is an optical response resulting in a change in the wavelength distribution patters or intensity of absorbance or fluorescence or a change in light scatter, fluorescence lifetime, fluorescence polarization, or a combination of the above parameters. Other detectable responses, include, for example, chemiluminescence, phosphorescence, radiation from radioisotopes, attraction to a magnet and electron density.

The term "directly detectable" as used herein refers to the presence of a detectable label or the signal generated from a detectable label that is immediately detectable by observation, instrumentation, or film without requiring chemical modifications or additional substances.

The term "enzyme" as used herein refers to a protein molecule produced by living organisms, or through chemical modification of a natural protein molecule, that catalyses chemical reaction of other substances without itself being destroyed or altered upon completion of the reactions. Examples of other substances, include, but are not limited to chemiluminescent, chromogenic, or fluorogenic substances.

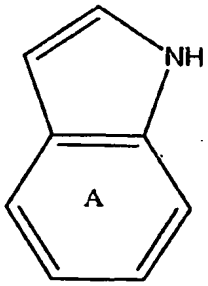
The term "halogen" as used herein refers to the substituents fluoro, bromo, chloro, and iodo.

The term "heteroaryl" as used herein refers to an aryl group as defined above in which one or more carbon atoms have been replaced by a non-carbon atom, especially nitrogen, oxygen, or sulfur. For example, such groups include furyl, tetrahydrofuryl, pyrrolyl, pyrrolidinyl, thienyl, tetrahydrothienyl, oxazolyl, isoxazolyl, triazolyl, thiazolyl, isothiazolyl, pyrazolyl, pyrazolidinyl, oxadiazolyl, thiadiazolyl, imidazolyl, imidazolinyl, pyridyl, pyridaziyl, triazinyl, piperidinyl, morpholinyl, thiomorpholinyl, pyrazinyl, piperainyl, pyrimidinyl, naphthyridinyl, benzofuranyl, benzothienyl, indolyl, indolinyl, indolizinyl, indazolyl, quinolizinyl, qunolinyl, isoquinolinyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, pteridinyl, quinuclidinyl, carbazolyl, acridinyl, phenazinyl, phenothizinyl, phenoxazinyl, purinyl, benzimidazolyl and benzthiazolyl. Many fluorophores are heteroaryl groups, including without limitations, xanthene, borapolyazaindacene, indole and quinazolone.

The above heterocyclic groups may further include on or more substituents at one or more carbon and/or non-carbon atoms of the heteroaryl group, e.g., alkyl; aryl; heterocycle; halogen; nitro; cyano; hydroxyl, alkoxyl or aryloxyl; thio or mercapto, alkyl- or arylthio; amino, alkyl-, aryl-, dialkyl-, diaryl-, or arylalkylamino; aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl or arylalkylaminocarbonyl; carboxyl, or alkyl- or aryloxycarbonyl; aldehyde; aryl- or alkylcarbonyl; iminyl, or aryl- or alkyliminyl; sulfo; alkyl- or arylsulfonyl; hydroximinyl, or aryl- or alkoximinyl. In addition, two or more alkyl substituents may be combined to form fused heterocycle-alkyl ring systems. Substituents including heterocyclic groups (e.g., heterocycleoxy, heteroaryloxy, and heteroaralkylthio) are defined by analogy to the above-described terms.

The term "heterocyclealkyl" refers to a heterocycle group that is joined to a parent structure by one or more alkyl groups as described above, e.g., 2-piperidylmethyl, and the like. The term "heterocyclealkyl" refers to a heteroaryl group that is joined to a parent structure by one or more alkyl groups as described above, e.g., 2-thienylmethyl, and the like.

The term "indole" or "indole derivative" as used herein refers to a compound generally having the formula:



Wherein the indole can be substituted or unsubstituted and can be attached to the BAPTA compound by a single covalent bond or as a fused ring wherein ring A of the indole also forms ring A or B of the BAPTA compound.

The term "isolated," as used herein with reference to the subject peptides, proteins and protein complexes, refers to, a preparation of peptide, protein or protein complex that is essentially free from contaminating proteins that normally would be present in association with the peptide, protein or complex, e.g., in a cellular mixture or milieu in which the protein or complex is found endogenously. In addition "isolated" also refers to the further separation from reagents used to isolate the peptide, protein or complex from cellular mixture. Thus, an isolated protein complex is isolated from cellular components and optionally from BAPTA compounds that normally would "contaminate" or interfere with the study of the complex in isolation, for instance while screening for modulators thereof.

The term "kit" as used refers to a packaged set of related components, typically one or more compounds or compositions.

The term "label" as used herein refers to a chemical used to facilitate identification and/or quantitation, either by itself or in combination with the remainder of the molecule, which it is attached. Illustrative labels include labels that can be directly observed or measured or indirectly observed or measured. Such labels include, but are not limited to, radiolabels that can be measured with radiation-counting devices; pigments, dyes or other chromogens that can be visually observed or measured with a spectrophotometer; spin labels that can be measured with a spin label analyzer; and fluorescent labels, where the output signal is generated by the excitation of a suitable molecular adduct and that can be visualized by excitation with light that is absorbed by the dye or can be measured with standard

fluorometers or imaging systems, for example. The label can be a luminescent substance such as a phosphor or fluorogen; a bioluminescent substance; a chemiluminescent substance, where the output signal is generated by chemical modification of the signal compound; a metal-containing substance; or an enzyme, where there occurs an enzyme-dependent secondary generation of signal, such as the formation of a colored product from a colorless substrate. The term label can also refer to a "tag" or hapten that can bind selectively to a labeled molecule such that the labeled molecule, when added subsequently, is used to generate a detectable signal. For example, one can use biotin as a tag and then use an avidin or streptavidin conjugate of horseradish peroxidate (HRP) to bind to the tag, and then use a chromogenic substrate (e.g., tetramethylbenzidine) or a fluorogenic substrate such as Amplex Red reagent (Molecular Probes, Inc.) to detect the presence of HRP. Numerous labels are know by those of skill in the art and include, but are not limited to, particles, fluorescent dyes, haptens, enzymes and their chromogenic, fluorogenic and chemiluminescent substrates and other labels that are described in RICHARD P. HAUGLAND, MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AD RESEARCH CHEMICALS (8th edition, CD-ROM, May 2001).

The term "labeled BAPTA" as used herein refers to a BAPTA compound that comprises one or more labels. Each label is independently attached to a ring by a single covalent bond or by a linker or shares two carbon atoms with the ring whereby it is a fused ring. A chemical moiety that in combination with the ring of the BAPTA to which it is attached facilitates identification and/or quantitation is also considered a label. An example of this is a fused benzofuran or quinazolone, as defined herein.

The term "metal chelator" as used herein refers to a chemical compound that combines with a metal ion to form a chelate ring structure.

The term "phosphorylated biomolecule" as used herein refers to a molecule possessing one or more phosphates each attached to such molecule by a single phosphoester bond. Phosphorylated molecules include, but are not limited to, phosphoproteins and phosphopeptides, phospholipids, phosphoglycans, phosphoamino acids, pyrophosphate and inorganic phosphate. Most known phosphate compounds, and subsequently the phosphorylated biomolecules, can be categorized into one of three groups; 1) single phosphate group (e.g. inorganic phosphate or phosphate on a protein); 2) multiple linked

phosphate group (e.g. pyrophosphate or ATP); or 3) bridging phosphate group (i.e. nucleic acids). For the purposes of the present invention phosphorylated biomolecules do not include molecules in the third group, e.g. DNA. Phosphoproteins and phosphopeptides are phosphorylated post-translationally on the tyrosine, serine or threonine amino acid residues. Thus, a phosphorylated protein or peptide comprises at least one of these amino acid residues. Phosphorylated biomolecules also include phosphorylated proteins, which can incorporate other non-peptide regions such as lipids, e.g. lipoproteins.

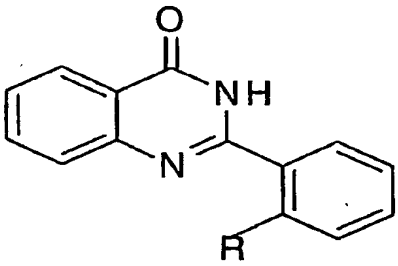
The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The term "sample" as used herein refers to any material that may contain phosphorylated biomolecules, natural or synthetic, as defined above. Typically, the sample comprises purified or semi-purified phosphorylated biomolecules. The phosphorylated biomolecules can be made synthetically or obtained in a purified or semi-purified form from cells (eukaryotic and prokaryotic, without limitation) cell extracts, cell homogenates, subcellular components as natural or recombinant molecules. Alternatively phosphorylated biomolecules can be obtained from tissue homogenate, bodily and other biological fluids, or synthesized proteins, all of which comprises a sample in the present invention.

The term "staining" as used herein refers to the formation of the tertiary complex of (un)labeled BAPTA, gallium ion and phosphorylated biomolecules.

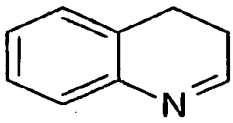
The term "tertiary complex" as used herein refers to the formation of BAPTA compound, gallium ions and phosphorylated biomolecule wherein gallium ions chelate BAPTA compounds and simultaneously bind phosphate groups on phosphorylated biomolecules forming a bridge between the two molecules.

The term "quinazolinone" or "quinazolinone derivative" as used herein refers to a compound generally having the structure:



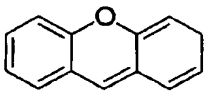
Wherein the quinazolinone can be substituted or unsubstituted, for example R can be an adjacent OH group. The quinazolinone can be attached to the BAPTA compound by a linker or a single covalent bond.

The term "quinoline" or "quinoline derivative" as used herein refers to a compound generally having the structure:



Wherein the quinoline can be substituted or unsubstituted at any carbon atom and is attached to a BAPTA compound by a linker or a single covalent bond.

The term "xanthene" or "xanthene derivative" as used herein refers to a compound generally having the formula:



Wherein the xanthene can be substituted or unsubstituted at any carbon atom and is attached to a BAPTA compound by a linker or a single covalent bond.

II. COMPOSITIONS AND METHODS OF USE

In accordance with the subject invention, methods and compositions are provided that stain phosphorylated biomolecules, such as lipids, proteins and peptides. Staining of phosphorylated biomolecules is accomplished when a sample containing phosphorylated biomolecules is combined with a mixture of gallium salt that comprises trivalent gallium ions, an acid and BAPTA compounds. The gallium ions form a bridge between the BAPTA compound and the phosphorylated biomolecule to form a tertiary complex. It is understood herein that staining of a phosphorylated biomolecule means the formation of the tertiary complex with a gallium ion chelating the BAPTA complex and concurrently binding to the phosphorylated biomolecule wherein this tertiary complex can be used without limitation in many and varied methods for the purposes of analyzing phosphorylated biomolecules, e.g. visualizing, isolating, precipitating and identifying phosphate groups on a molecule. The phosphorylated biomolecules can then be further isolated or visualized when the BAPTA compound optionally comprises a label that is detectable or functions as a hapten.

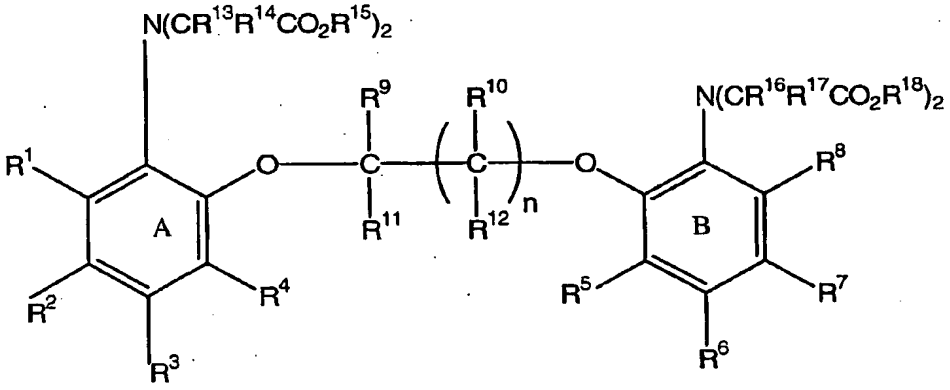
In general, for ease of understanding the present invention, the components of the staining mixture will first be described in detail, followed by the many and varied methods in which the tertiary complex finds use, which is followed by exemplified methods of use and synthesis of novel compounds that are advantageous for use with the methods of the present invention.

To practice the methods of the invention, a sample is combined with a (un)labeled BAPTA compound and a gallium salt in an acidic environment. The staining mixture can be prepared in a variety of ways, which are dependent on the medium the sample is in, as described in detail below. Specifically the staining mixture comprises an (un)labeled BAPTA compound, a gallium salt and an acid in an aqueous solution. The resulting tertiary complex is stable in an acidic environment, which is advantageous for many of the methods of the present invention.

A large number of chelating moieties on the BAPTA compounds that might form stable tertiary complexes with gallium ions and phosphorylated compounds were examined. However, it was found that certain BAPTA compounds were the only chelating moieties that resulted in stable, detectable tertiary complexes. These BAPTA compounds exhibited sufficient binding affinity for the gallium (III)-phosphorylated compound complex to allow for rinsing away of excess reagents from the persistent tertiary complex, whereby detection

did not need to be dependent on an increase in detectable signal from the formation of the tertiary complex. Additionally, it was found that certain labeled BAPTA compounds provided optimal signal after formation of the tertiary complex. This high signal is a function of well-tuned hydrophobicity of the labeled BAPTA-gallium (III)-phosphorylated compound complex and well-tuned binding affinity of the labeled BAPTA compound. Following is a detailed description of the preferred BAPTA compounds of the present invention that find particular use in the formation of the tertiary complex.

The BAPTA compounds of the present invention bind metal ions with high affinity and preferred ions for the staining of phosphorylated biomolecules are iron, aluminum and gallium, most preferred is trivalent gallium ions present in a gallium salt (Example 1). Preferred BAPTA compounds used to practice the invention have Formula 1:



Preferably ring A and ring B are linked by a hydrocarbon bridge between two oxygen atoms in which n is 1 or 2 and the ring substituents (R^1-R^8) are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicylic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano. Alternatively, two adjacent substituents in combination constitute a cyclic substituent that is alkyl, heteroalkyl, aryl and heteroaryl. Preferably, a BAPTA compound has at least two substituents that are not hydrogen, a most preferred BAPTA compound for the formation of the tertiary complex is a BAPTA compound with a fluorine atom as one of the substituents, most preferably substituted at the R^6 or R^3 position (e.g. compound 1, 3, 5, 6, 8 and 9). Equally preferred are BAPTA compounds that comprise a carbonyl group as a substituent, preferably at the R^7 position, e.g. compound 9 and 12.

Without being bound by a particular theory, it appears that an electron withdrawing group such as fluorine or carbonyl substituted at the R^3, R^4, R^6 or R^7 position results in BAPTA compounds that are particularly advantageous for chelating gallium ions that then also allows for the simultaneous interaction of the chelated gallium ion with a phosphate group on the phosphorylated biomolecule resulting in a stable tertiary complex.

The bridge substituents R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents R^9 and R^{10} in combination constitute, a 5-membered or 6-membered alicyclic or heterocyclic ring. R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy, preferably R^{13}, R^{14}, R^{16} and R^{17} are hydrogen. R^{15} and R^{18} are independently hydrogen or a salt.

The following references describe illustrative BAPTA compounds that can be utilized with the methods of the invention: U.S. Pat. No. 4,603,209 (1986) to Tsien *et al.*; Tsien, "New calcium indicators and Buffers with high selectivity against magnesium and protons: Design, synthesis and properties of protype structures" Biochem. 19:2396-2404 (1980); U.S. Pat. Nos. 4,849,362; 5,049,673; 5,453,517; 5,459,276; 5,516,911; 5,501,980; 5,773,227.

Alternatively, the BAPTA compounds comprise one or more labels that generate a detectable response. The detectable response can be generated solely from a substituent that forms one of the R groups, preferably R^1-R^8, that is not part of the BAPTA compound as defined in Formula 1 or the detectable response can be generated from the combination of a prolabel and the BAPTA compound, wherein the prolabel is defined as a precursor or a portion of label that when covalently combined with the BAPTA compound is capable of generating a detectable response. The prolabel can be attached to the BAPTA compound by a single covalent bond, by a linker or fused to a ring so that the prolabel and BAPTA share one or more carbon atoms. Therefore, when a prolabel is attached to a BAPTA compound, a complete label moiety is formed that is capable of generating a detectable response.

Labels of the invention comprise dyes or other chromogens, fluorescent dyes, enzymes, haptens, particles, pigments, spin labels, radioisotopes and any moiety capable of generating a detectable response. Preferably the label is a fluorescent dye, an enzyme, or a hapten. More preferably the label is a fluorescent dye or a hapten. Equally preferred is a labeled

BAPTA compound comprising one or more fluorescent dyes wherein the BAPTA is optionally attached to biotin on the adjacent ring.

A dye label (fluorophore or chromophore) is any chemical moiety that exhibits an absorption maximum beyond 280 nm, that is bound to the BAPTA compound by a covalent linkage L, or that is fused to the BAPTA compound, as defined above. The covalent linkage L is either a single covalent bond, or a combination of stable chemical bonds, as described in greater detail below. The covalent linkage binding the dye label to the BAPTA compound is typically a single bond or a fused ring, but optionally incorporates 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, and P. For example, L is or incorporates a phenylene or a 2-carboxy-substituted phenylene.

Typically the dye label contains one or more aromatic or heteroaromatic rings, that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on chromophores or fluorophores known in the art.

In one aspect of the invention, the dye label has an absorption maximum beyond 480 nm. In a particularly useful embodiment, the dye label absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp). The dye label may be a chromophore, resulting in a compound that acts as a chromogenic stain for phosphorylated biomolecules, or more preferably, the dye label is additionally a fluorophore, resulting in a compound that is a fluorescent stain. Preferably, binding a gallium ion within the BAPTA compound and the gallium ion concurrently binding a phosphorylated biomolecule results in a detectable optical response. As used herein, a detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or instrumentally, such as a change in absorption (excitation) wavelength, fluorescence emission wavelength, or fluorescence emission intensity.

For an application where a detectable response is desirable, e.g. staining phosphorylated biomolecules in solution, and where the detectable response is a fluorescence response, it is typically a change in fluorescence, such as a change in the intensity, excitation or emission

wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. Preferably, the detectable optical response upon binding the gallium ion and phosphorylated biomolecule is a change in fluorescence intensity that is greater than approximately 10-fold, more preferably greater than 50-fold. In another aspect, the detectable optical response upon binding the gallium ion phosphorylated biomolecule complex is a shift in maximal excitation or emission wavelength that is greater than about 20 nm, more preferably greater than about 30 nm. Alternatively, for applications wherein the phosphorylated biomolecule or BAPTA compound is immobilized resulting in an immobilized tertiary structure, an increase in detectable fluorescence response due to the chelation of the BAPTA compound and subsequent tertiary complex formation is not necessary. This is due to the stable tertiary complex, which allows for washing and removal of unbound BAPTA compounds wherein the fluorescent response from the BAPTA compound is enough to visualize the phosphorylated biomolecule.

A wide variety of chemically reactive fluorescent dyes that may be suitable for incorporation into the BAPTA compounds of the invention are already known in the art (see for example MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, 8th Edition, CD-ROM).

Typical dye labels of the invention are a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, (as described in U.S. Patent No. 5,830,912), a polyazaindacene (e.g., US Patent Nos. 4,774,339; 5,187,288; 5,248,782; 5,274,113; and 5,433,896), a xanthene, an oxazine or a benzoxazine, a carbazine (US Patent No. 4,810,636), or a phenalenone or benzphenalenone (US Patent No. 4,812,409). Other typical dye labels are a carbazine, an oxazine, a pyrene, a xanthene, a naphthalene, a phenalenone, or a polyazaindacene. As used herein, oxazines include resorufins, aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

Where the dye label is a xanthene, the synthetic dye is optionally a fluorescein, a rhodol (US Patent No. 5,227,487), or a rhodamine. As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (U.S. Patent No. 4,945,171). Fluorinated

xanthene dyes have been described previously as possessing particularly useful fluorescence properties (Int. Publ. No. WO 97/39064 and U.S. Patent No. 6,162,931).

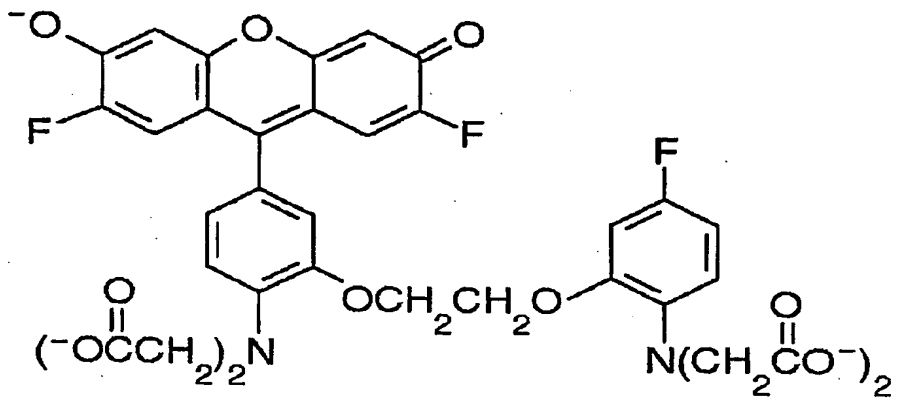
Alternatively, the dye label is a xanthene that is bound via an L that is a single covalent bond at the 9-position of the xanthene. Preferred xanthenes include derivatives of 3*H*-xanthen-6-ol-3-one attached at the 9-position, derivatives of 6-amino-3*H*-xanthen-3-one attached at the 9-position, or derivatives of 6-amino-3*H*-xanthen-3-imine attached at the 9-position.

The most preferred fluorescent dyes of the present invention for generating a strong detectable signal and facilitating formation of the tertiary complex include benzofuran, quinoline, quinazolone, xanthene, benzazole, and borapolyazaindacene including any derivatives thereof. These fluorescent dyes all produce a strong detectable signal when the dye comprises a BAPTA compound. It is an important aspect of the current invention that none of the preferred fluorescent dyes are sulfonated.

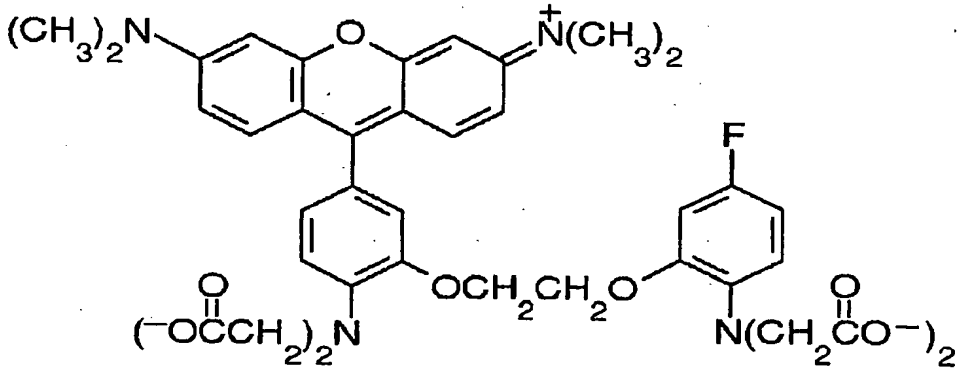
As described above, two adjacent substituents of R^1-R^8, when taken in combination with each other, and with the aromatic ring they are bound to, form a fused label. In one aspect of the invention, the fused label is a carbocyanine dye (as in Wang *et al.*, J. WUHAN UNIV 2, 73 (1991)). In another aspect of the invention, two adjacent ring substituents taken in combination form the dye label that is a fused benzofuran or heteroaryl- or carboxyheteroaryl-substituted benzofuran fluorophore, or a fused 6-membered unsaturated lactone, or benzazole-substituted lactone. Where the dye label is fused to the compound of the invention, it is preferably fused R^2 and R^3, or at R^6 and R^7.

Especially preferred fluorescent dye labeled BAPTA compounds are:

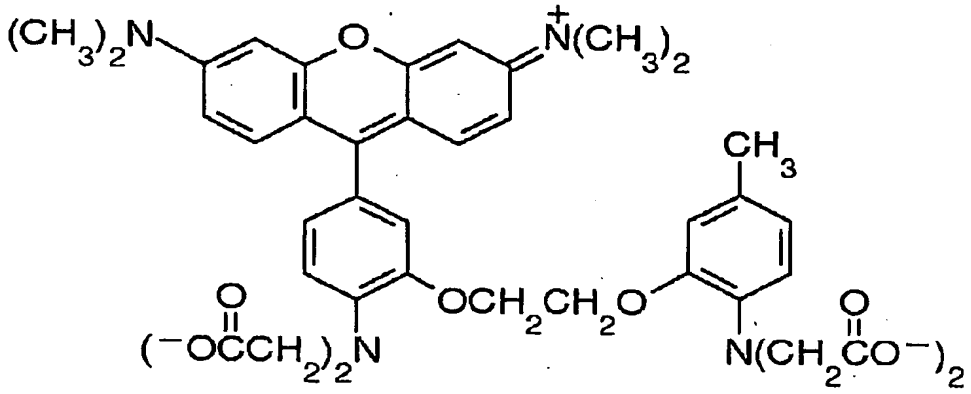
Compound 1, wherein the dye label is a fluorinated hydroxy xanthenone and the BAPTA component is fluorinated at the R^6 position.



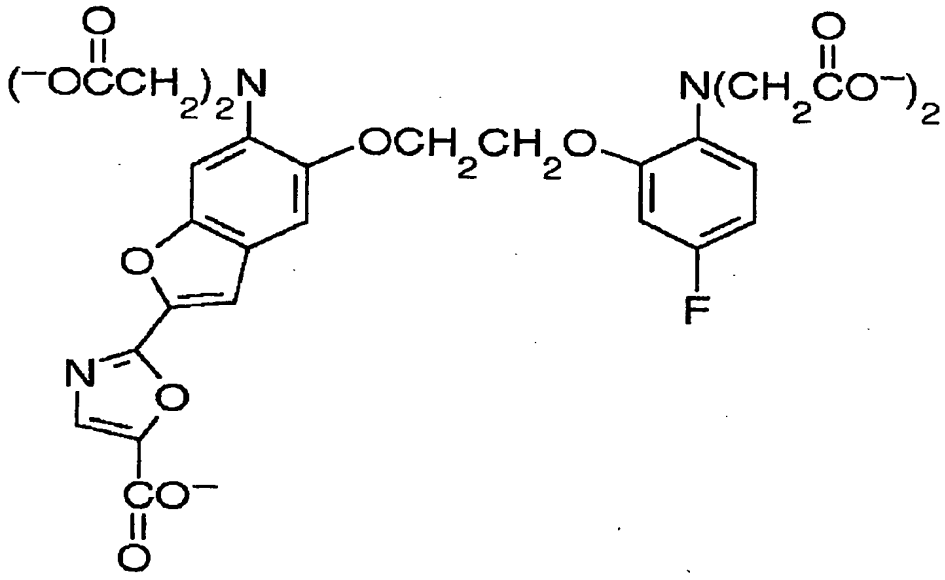
Compound 2, wherein the dye label is an amino/imino xanthene and the BAPTA component is fluorinated at the R^6 position.



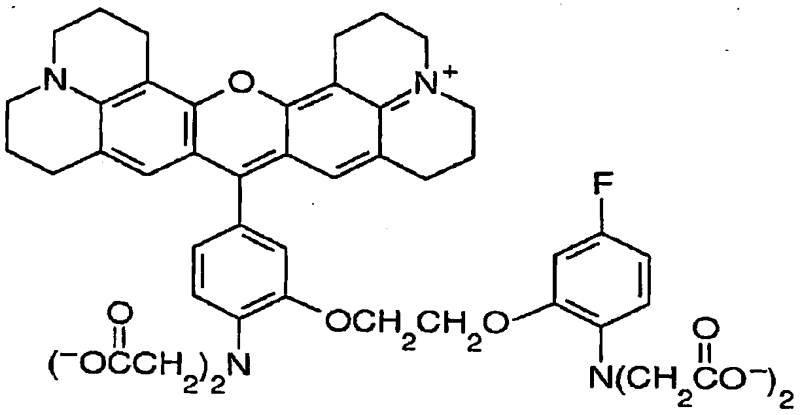
Compound 3, wherein the dye label is an amino/imino xanthene.



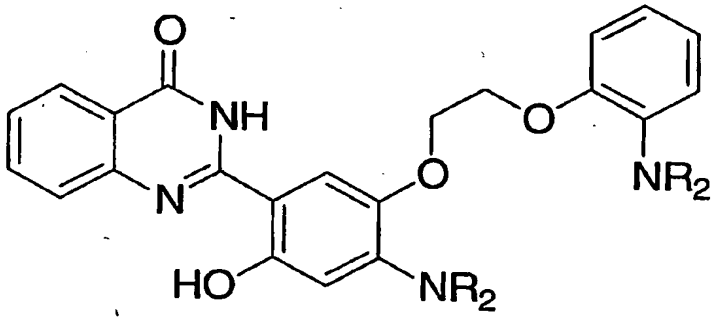
Compound 4, wherein the dye label is a fused benzofuran with a substituted heteroaryl and the BAPTA component is fluorinated at the R^6 position.



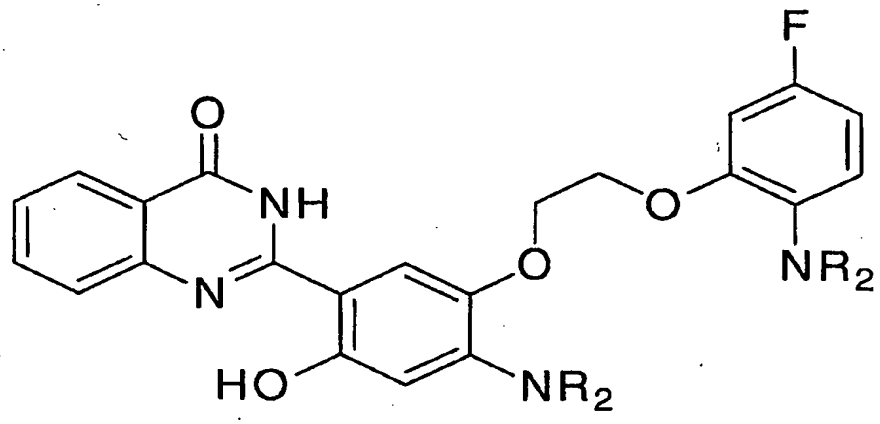
Compound 5, wherein the dye label is a julolidene xanthene and the BAPTA component is fluorinated at the R^6 position.



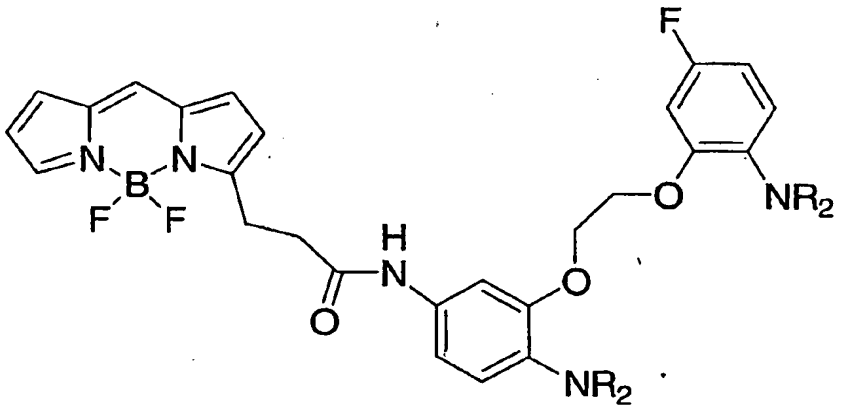
Compound 6, wherein R is CH_2CO_2K and the dye label is quinazolinone with an adjacent hydroxyl group.



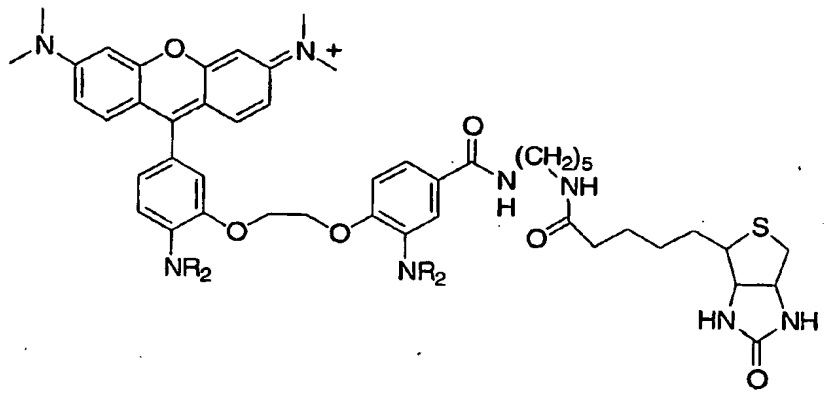
Compound 7, wherein R is CH_2CO_2K and the dye label is a quinazolinone with an adjacent hydroxyl group and the BAPTA compound is fluorinated at the R^6 position.



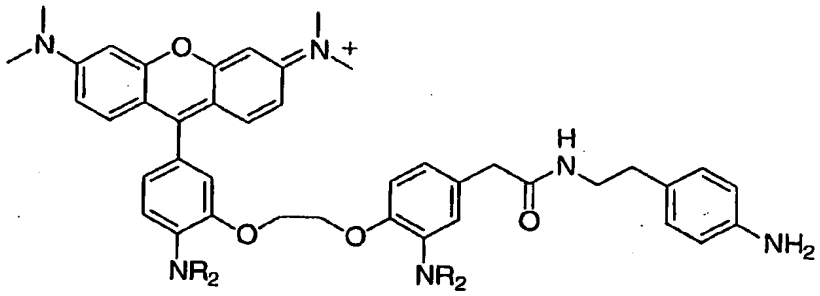
Compound 8, wherein R is CH_2CO_2Na, the dye label is borapolyazaindacene and the BAPTA component is fluorinated at the R^6 position.



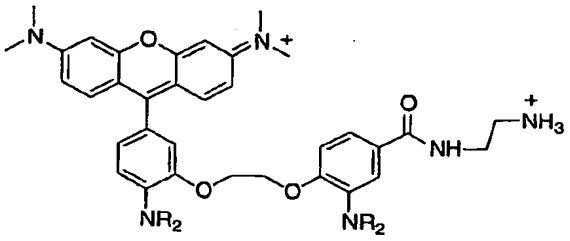
Compound 9, wherein R is CH_2CO_2K, the dye label is xanthene and the BAPTA component is substituted by a carbonyl group at the R^7 position that is attached to a biotin by a linker.



Compound 10, wherein R is CH_2CO_2K, the dye label is xanthene and the BAPTA component is substituted by aniline at the R^7 position.



Compound 11, wherein R is CH_2CO_2K, the dye label is a xanthene and the BAPTA component is substituted by a carbonyl group at the R^7 position.



In addition to dyes, enzymes also find use as labels for BAPTA compounds. Enzymes are preferred labels or prolabels because amplification of the detectable signal can be obtained resulting in increased assay sensitivity. The enzyme itself does not produce a detectable response but functions to break down a substrate when it is contacted by an appropriate substrate such that the converted substrate produces a fluorescent, chromogenic or luminescent signal. Enzymes amplify the detectable signal because one enzyme on a BAPTA compound can result in multiple substrates being converted to a detectable signal. This is advantageous where there is a low quantity of phosphorylated biomolecules present in the sample or a fluorescent dye does not exist that will give comparable or stronger signal than the enzyme. Fluorescent dyes are most preferred because they do not require additional assay steps. The enzyme substrate is selected to yield the preferred measurable product, e.g. chromogenic, fluorescent or chemiluminescence. Such substrates are extensively used in the art, many of which are described in the MOLECULAR PROBES HANDBOOK, *supra*.

A preferred chromogenic or fluorogenic substrate and enzyme combination uses oxidoreductases such as horseradish peroxidase and a substrate such as 3,3'-diaminobenzidine (DAB) and 3-amino-9-ethylcarbazole (AEC), which yield a distinguishing color (brown and red, respectively). Other chromogenic oxidoreductase substrates that yield detectable products include, but are not limited to: 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), *o*-phenylenediamine (OPD), 3,3',5,5'-tetramethylbenzidine (TMB), *o*-dianisidine, 5-aminosalicylic acid, 4-chloro-1-naphthol. Fluorogenic substrates include, but are not limited to, homovanillic acid or 4-hydroxy-3-methoxyphenylacetic acid, reduced phenoxazines and reduced benzothiazines, including Amplex Red™ reagent and its variants (U.S. Pat. No. 4,384,042) and reduced dihydroxanthenes, including dihydrofluoresceins such as those described in U.S. Pat. No. 6,162,931 and dihydrorhodamines such as dihydrorhodamine 123. Peroxidase substrates that are tyramides, as in U.S. Pat. Nos.

5,196,306; 5,583,001 and 5,731,158, represent a unique class of peroxidase substrates in that they can be intrinsically detectable before action of the enzyme but are "fixed in place" by the action of a peroxidase in the process described as tyramide signal amplification (TSA). These substrates are extensively utilized to label targets in samples that are cells, tissues or arrays for their subsequent detection by microscopy, flow cytometry, optical scanning and fluorometry.

Another preferred chromogenic (and in some cases fluorogenic) substrate and enzyme combination uses a phosphatase enzyme such as calf intestinal alkaline phosphatase, an acid phosphatase, or a recombinant version of such a phosphatase in combination with a chromogenic substrate such as 5-bromo-6-chloro-3-indolyl phosphate (BCIP), 6-chloro-3-indolyl phosphate, 5-bromo-6-chloro-3-indolyl phosphate, *p*-nitrophenyl phosphate, or *o*-nitrophenyl phosphate or with a fluorogenic substrate such as 4-methylumbelliferyl phosphate, 6,8-difluoro-7-hydroxy-4-methylcoumarinyl phosphate (DiFMUP, U.S. Pat. No. 5,830,912) fluorescein diphosphate, 3-*O*-methylfluorescein phosphate, resorufin phosphate, *9H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (DDAO phosphate), or ELF 97, ELF 39 or related phosphates (U.S. Pat. Nos. 5,316,906 and 5,443,986).

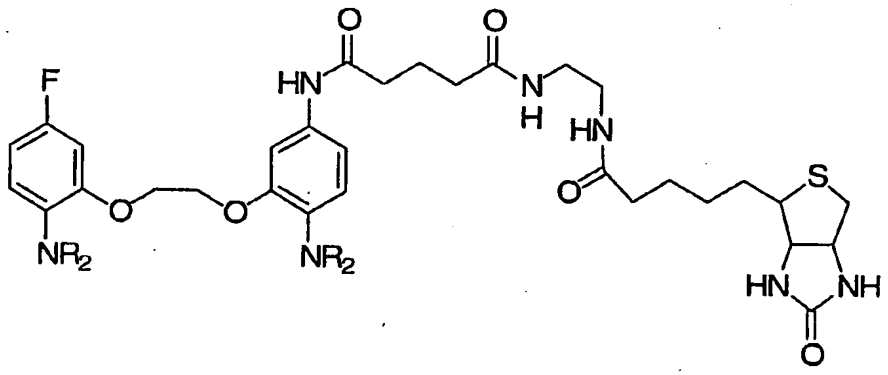
Glycosidases, in particular beta-galactosidase, beta-glucuronidase and beta-glucosidase, are additional suitable enzymes. Appropriate chromogenic substrates include, but are not limited to, 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (X-gal) and similar indolyl galactosides, glucosides, and glucuronides, *o*-nitrophenyl beta-D-galactopyranoside (ONPG) and p-nitrophenyl beta-D-galactopyranoside. Preferred fluorogenic substrates include resorufin beta-D-galactopyranoside, fluorescein digalactoside (FDG), fluorescein diglucuronide and their structural variants (U.S. Pat. Nos. 5,208,148; 5,242,805; 5,362,628; 5,576,424 and 5,773,236), 4-methylumbelliferyl beta-D-galactopyranoside, carboxyumbelliferyl beta-D-galactopyranoside and fluorinated coumarin beta-D-galactopyranosides (U.S. Pat. No. 5,830,912).

Additional enzymes include, but are not limited to, hydrolases such as cholinesterases and peptidases, oxidases such as glucose oxidase and cytochrome oxidases and reductases for which suitable substrates are known.

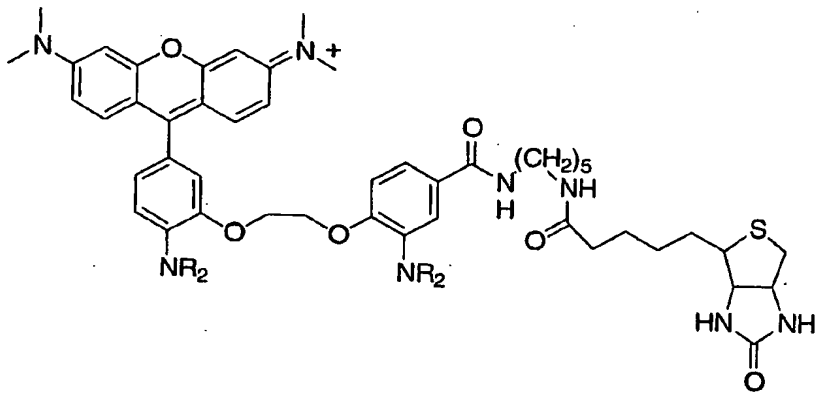
Enzymes and their appropriate substrates that produce chemiluminescence are preferred for some assays. These include, but are not limited to, natural and recombinant forms of luciferases and aequorins. Chemiluminescence-producing substrates for phosphatases, glycosidases and oxidases such as those containing stable dioxetanes, luminol, isoluminol and acridinium esters are additionally useful.

In addition to enzymes, haptens such as biotin are also preferred labels. Biotin is useful because it can function in an enzyme system, to further amplify the detectable signal, and it can function as a tag to be used in affinity chromatography for isolation purposes. A BAPTA compound labeled with a fluorescent dye and optionally a biotin is useful for removing the BAPTA compound from the phosphorylated protein or peptide after isolation, described in more detail below.

A preferred biotinylated BAPTA compound is:
Compound 12, wherein, R is CH_2CO_2K, the BAPTA component is fluorinated at the R^3 position and substituted by an amine group at the R^6 position that is attached to a biotin by a linker.



A preferred BAPTA compound with a dual label of biotin and fluorescent dye is Compound 9.



Synthesis of preferred BAPTA compounds

The synthetic strategy of labeled BAPTA compounds that provide optimal signal after formation of the tertiary complex involves selection of appropriate chemical linkages between the label(s) and the BAPTA moiety, and also selection of appropriate substituents on the BAPTA moiety. Such selections are made so that the resulting labeled BAPTA compound retains optimal binding affinity and solubility to promote a persistent tertiary complex. Improper selections result in labeled BAPTA compounds that do not have sufficient binding affinity and do not produce a persistent tertiary complex. Improper selections also result in excessive non-selective binding of the labeled BAPTA compound to analytes other than the target phosphorylated compounds, resulting in a high background and thus a low signal-to-noise ratio.

The novel BAPTA compounds of the present invention include BAPTA compounds with a quinazolinone fluorescent label (compound 6, 7 and 15), BAPTA compounds with a borapolyazaindacene fluorescent dye (compound 8 and 16), BAPTA compounds with a biotin label wherein the biotin is attached by a linker (compound 9, 12 and 18), a BAPTA compound with benzothiazole label (compound 17), BAPTA compounds with agarose covalently attached (compound 13 and 14) and BAPTA compounds comprising an aniline attached by a linker to the BAPTA compound (compound 10). These novel BAPTA

compounds find use in the staining and isolation of phosphorylated biomolecules. Synthesis of these compounds is exemplified in Examples 18-24.

Sample preparation

The sample of the present invention contains or is suspected to contain phosphorylated biomolecules, as defined above. The sample optionally further comprises an aqueous solution, typically prepared with water (e.g. for pure proteins or peptides) or aqueous buffer, or is combined with an aqueous solution in the course of staining.

Depending on the source of the sample mixture, it optionally contains discrete biological ingredients other than the desired phosphorylated biomolecules, including proteins, peptides, lipids, amino acids, nucleic acids and carbohydrates, which may or may not be removed in the course of, prior to, or after staining. The desired phosphorylated biomolecules and other discrete biological components can be optionally separated from each other or from other components in the sample by mobility (e.g. electrophoretic gel or capillary) by size (e.g. centrifugation, pelleting or density gradient), or by binding affinity (e.g. to a filter membrane or affinity resin) in the course of the present methods. For certain aspects of the invention it is preferred that the phosphorylated biomolecules not be separated.

When starting with a sample source that is not appropriate for separation, e.g. whole cells or tissue homogenate, the sample needs to first be prepared using techniques well known to those skilled in the art. Preparation of the sample will depend on how the phosphorylated biomolecules are contained in the sample (*See* e.g., Current Protocols in Molecular Biology; Herbert, *Electrophoresis 20*:660-663 (1999)). For example, an optional way of preparing samples for 2D gel electrophoresis followed by staining with the compositions and methods of the present invention includes lysing cells using a lysis buffer that ensures that the proteome, in addition to post-translational modifications, of a sample remain in their *in vivo* state throughout the entire procedure. Examples of such buffers include ones derived from a thiourea/NP-40/DTT mixture as described in O'Farrell, *J. Biol. Chem. 250*:4007-4021 (1975). Therefore, the lysis buffer might additionally contain phosphatase inhibitors such as sodium orthovanadate, sodium fluoride or β-glycero-phosphate in addition to a protease inhibitor cocktail.

Typically the phosphorylated proteins in the sample have a molecular weight greater than about 500 daltons. More typically the phosphorylated proteins are more than 800 daltons. In one aspect of the invention the phosphorylated proteins comprise a mixture of phosphorylated proteins with different molecular weights that fall within a range of molecular weights, wherein the phosphorylated proteins are used as molecular weight standards so that stained phosphorylated proteins or peptides can be accurately analyzed. Samples comprising phosphorylated peptides subjected to the methods of the present invention can be generated from natural or synthetic samples and may be the result of chemical, physical or enzymatic digestion of phosphorylated protein samples. Proteins can be digested using any appropriate enzymatic method, such as trypsin digestion. Peptides in the digest may be preferably sized to facilitate peptide sequencing using tandem mass spectrometric methods, and are typically in the size range from about 10 to about 50 amino acids in length.

Samples comprising phospholipids wherein the phospholipids are to be stained are prepared with modification compared to samples comprising phosphoproteins prior to applying to solid or semi-solid matrix due to their hydrophobic nature. Most samples typically require some sort of extraction treatment prior to staining with the compositions and methods of the present invention. Where the phosphorylated biomolecule of interest come from tissue samples or samples from organisms having cell walls, mechanical or chemical disruption may be required. Suitable means are well known in the art and include, but are not limited to, the use of a tissue homogenizer or a French pressure cell in conjunction with, for example, organic solvent extractions. Methods of cell disruption and fractionation are described in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons 1997). Samples may be extracted with solvents possessing varying hydrophobic properties, and the optimal solvent is contingent upon the nature of the phosphorylated biomolecule of interest. Extraction techniques conventional in the art that result in a sample suitable for detection are contemplated by the present invention.

The following references describe various extraction techniques: Dole *et al., J. Clin. Invest. 35*:150-54 (1956); Dole *et al., J. Biol. Chem. 235*:2595-99 (1960); Bligh *et al., Canadian J. Biochem. Physiol. 37*:914-17 (1959); Folch *et al., J. Biochem. 226*:497-509 (1957). The Dole *et al.* references describe an extraction method that involves extractions of the sample with an isopropanol/heptane/sulfuric acid mixture followed by several heptane extractions. The organic phase is dried with nitrogen for use in subsequent steps. The Folch *et al.* reference

describes the extraction of lipids from biological tissue homogenates or body fluids. Samples are extracted with chloroform/methanol, filtered and reverse-extracted with 0.1 M KCl. The Bligh *et al.* reference describes the organic extraction of lipids from biological tissue homogenates or fluids. Samples are extracted with methanol/chloroform and chloroform, and then filtered and reverse-extracted with water.

Typically, the phosphorylated biomolecules (proteins, peptides or lipids) are present on or in a solid or semi-solid matrix. In one aspect of the invention, this matrix comprises an electrophoresis medium, such as a polyacrylamide gel, agarose gel, linear polyacrylamide solution or polyvinyl alcohol gel. The solid or semi-solid matrix can also comprise a membrane, such as a filter membrane, a nitrocellulose, poly(vinylidene difluoride) (PVDF) membrane, or nylon membrane wherein the phosphorylated biomolecules are immobilized on the membrane by blotting, spotting, electroblotting (tank and semi-dry), capillary blotting or other methods of application well known to those skilled in the art. In accordance with the present invention solid and semisolid matrix also includes a glass slide, a plastic matrix (e.g. multi-well plate or pin), a glass or polymeric bead or semiconductor material. The phosphorylated biomolecules may be arrayed on the support in a regular pattern or randomly. A preferred array of the present invention is a glass support wherein the phosphorylated biomolecules of the sample are arrayed in a regular pattern. For certain aspects of the invention it is preferred that the phosphorylated biomolecules be free from a solid or semi-solid matrix, i.e. not immobilized and present in an aqueous solution as solubilized solubilize molecules.

Staining Mixture

The staining mixture can be prepared in a variety of ways, which are dependent on the medium the sample is in, as described below. Specifically the staining mixture comprises an (un)labeled BAPTA compound, a gallium salt and an acid in an aqueous solution; optionally the staining mixture comprises an organic solvent and a buffering component, e.g. sodium chloride. Any of the components of the staining mixture can be added together or separately and in no particular order. The BAPTA compound is prepared by dissolving in a solvent, such as water, DMSO, DMF or methanol, usually at a final concentration of about 0.1 μM to 10 μM, preferably the BAPTA compound is present in the staining mixture at a concentration of about 0.5 μM to 5 μM and most preferred is a concentration about 1.0 μM. However, for a

staining mixture useful for precipitating phosphorylated biomolecules from solution a higher concentration of BAPTA is desired, preferably about 0.05mM to 1mM.

The gallium salt preferably contains trivalent gallium ions, such as gallium chloride, but can be any gallium salt known to those skilled in the art. Gallium salts that can be used with the present invention include, without limit, acetylacetonate, arsenide, bromide, fluoride, iodide, nitrate, nitride, perchlorate, sulfate and sulfide. The gallium salt is typically present in the staining mixture at a concentration of about 10nm to 1mM; preferably the concentration is about 0.5μM to 10μM. However for precipitation purposes the gallium salt is preferably present at a slightly higher concentration of about 0.5mM to 0.1mM.

Analysis of the stability and specificity of the BAPTA compounds for gallium ions and the gallium ions for the phosphorylated biomolecules was evaluated as a function of pH (Example 1). Therefore, a preferred staining mixture comprises an acid to provide an acidic environment for the staining reaction. An acidic environment is defined as a solution having a pH less than 6.9. Typical suitable acidic components include without limitation acetic acid, trichloroacetic acid, trifluoroacetic acid, perchloric acid, phosphoric acid, or sulfuric acid. The acidic component is typically present at a concentration of 1%-20%. The pH of the staining mixture is preferably about pH 3-5 and most preferred is about pH 4.0.

The pH of the staining mixture is optionally modified by the inclusion of a buffering agent in addition to or in place of an acidic component. In particular, the presence of a buffering agent has been shown to improve staining of electrophoresis gels, provided that an alcohol is included in the formulations as well. Any buffering agent that maintains an acidic environment and is compatible with the phosphorylated biomolecules in the sample is suitable for inclusion in the staining mixture.

Useful buffering agents include salts of formate, acetate, 2-(N-morphilino) ethanesulfonic acid, imidazole, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid, Tris (hydroxymethyl)aminomethane acetate, or Tris (hydroxymethyl)aminomethane hydrochloride wherein the buffering agent does not chelate gallium ions. An exemplified buffering agent is sodium acetate. The buffering agent is typically present in the staining mixture at a concentration of about 20 mM to 500 mM; preferably the concentration is about 50mM to 200 mM.

Inclusion of a polar organic solvent, typically an alcohol, in the staining mixture is recommended wherein the staining mixture contains a pH buffering agent and a salt. While the use of highly polar solvents such as formamide is permitted, typically, the polar organic solvent is an alcohol having 1-6 carbon atoms, or a diol or triol having 2-6 carbon atoms. A preferred alcohol is 1,2-propanediol. The polar organic solvent, when present, is typically included in the staining mixture at a concentration of 5-50%. The presence of a polar organic solvent is particularly advantageous when staining SDS-coated proteins, as is typically the case when staining phosphorylated proteins or peptides that have been electroblotted from SDS-polyacrylamide gels. Typically SDS is removed from a gel or blot prior to staining by fixing and washing, however some SDS may remain and interferes with the staining methods of the present invention. Without wishing to be bound by any theory, it appears that the presence of an alcohol improves luminescent staining of phosphorylated proteins or peptides by removing any SDS that was not removed by the washing or fixing. However, nitrocellulose membranes may be damaged by high concentrations of alcohol (for example, greater than 20%), and so care should be taken to select solvent concentrations that do not damage the membranes that the phosphorylated proteins or peptides are immobilized upon.

Staining of immobilized phosphorylated biomolecules (e.g., in a gel, on a blot or on an array)

The staining mixture is combined with the sample in such a way as to facilitate contact between the (un)labeled BAPTA compound/gallium ion complex and any phosphorylated biomolecules present in the combined mixture wherein formation of a tertiary complex effectively stains present phosphorylated biomolecules. For phosphorylated biomolecules immobilized on a surface the stain is typically incubated with the support the biomolecules are immobilized on, i.e. the gel or blot is incubated with stain, under conditions which maximize contact such as gentle mixing or rocking.

As mentioned above SDS interferes with the staining methods of the present invention, therefore SDS needs to be removed prior to addition of the staining mixture. Gels and blots are fixed and washed, which results in the removal of SDS from the gels or blots. A preferred fixing mixture for gels and blots comprises methanol and acetic acid; optionally the fixing mixture comprises glutaraldehyde. The methanol is present at a concentration of about 35-50% and the acetic acid is present at about 0-15% and the gluteraldehyde is present at about 0-2%. Typically, fixing is followed by washing the gels or blots in 100% water.

Therefore, after samples have been separated on a gel or transferred to a polymeric membrane, fixed, and washed the gel or blot is incubated with a staining mixture (Examples 2-9). The phosphorylated proteins or peptides are incubated with the staining mixture for a time sufficient for the gallium ion/BAPTA complex to bind to the present phosphorylated proteins or peptides. Preferably this time is not more than 24 hours, more preferably this time is less than 8 hours and most preferably this incubation time is less than 2 hours but not less than 5 minutes. After incubation with the staining mixture the gels or blots are washed with a mixture that preferably comprise an acidic buffering agent; useful buffering agents to be used with the present invention include, without limitation, sodium acetate, and formate and 2-(N-morpholino)ethanesulfonic acid. Typically, the buffering agent is present in the washing solution at a concentration of about 25mM to about 100 mM. Thus, following staining and washing the trivalent complex can then be illuminated directly when the BAPTA compound is labeled as described above to visualize the phosphorylated biomolecules. Preferably the BAPTA compounds are labeled but optionally they may be unlabeled and detected with a labeled anti-BAPTA antibody or anti-fluorophore antibody, which is then illuminated indicating staining of the phosphorylated biomolecules.

The BAPTA compounds comprising the staining mixture are chosen depending on their ability to bind phosphorylated biomolecules in different mediums. Therefore, preferred BAPTA compounds for staining phosphorylated biomolecules in a gel are compounds 1-4 and 7-11 of the present invention. Preferred compounds for staining phosphorylated biomolecules on a blot are compounds 1, 4 and 7 of the present invention.

A particular advantage to identifying phosphorylated proteins or peptides in a 2-D gel is the ability to correctly identify the phosphoproteome as well as quantitate post-translational modification of proteins for the addition or subtraction of phosphate groups. Specifically staining for phosphorylated proteins or peptides while doing concurrent, or subsequent, total protein staining identifies the phosphorylated proteome while the intensity of the signal can be correlated to the level of phosphorylation, when compared to the total protein stain (Examples 6, 7 and 13). Any fluorescent dye specific for total proteins can be used to stain total proteins in the gel; a preferred stain is SYPRO™ Ruby dye for gels or any dye disclosed in US Patent No. 6,316,276 B1. Because SDS is removed by washing prior to staining with the staining mixture of the present invention total protein stains such as SYPRO™ Ruby are preferred because SDS is not critical for the staining function. However, protocol changes

can be made when using a stain that requires SDS for staining sensitivity, such as SYPRO™ Orange and SYPRO™ Red, by adding SDS back to the gel prior to a total protein stain step and including SDS in the staining mixture (Malone *et al.* Electrophoresis (2001) 22(5):919-32). A preferred mixture for returning SDS back to a gel is 2% acid/0.0005% SDS, and optionally 40% ethanol, wherein the gel is incubated for at least one hour. Alternatively the total protein stain could be preformed prior to the phosphorylated staining of the present invention; therefore the SDS would not need to be added back to the gel but simply removed prior to phosphorylated staining as contemplated by the present invention. Therefore, alternative preferable total protein stains for gels are SYPRO™ Orange, SYPRO™ Tangerine and SYPRO™ Red or any dye disclosed in US Patent No. 5,616,502 or U.S. Serial No. 09/632,927. Alternative total protein stains for gels include Coomassie Blue or silver staining, staining techniques well known to those skilled in the art. Alternative total proteins stains useful for staining blots are SYPRO™ Rose Plus and DyeChrome™ or any stain disclosed in US Patent No. 6,329,205 B1 and US Serial No. 10/005,050.

Another, very important advantage when staining phosphorylated biomolecules in a 2-D gel is to include a stain for glycoproteins wherein a 3-way analysis of the proteome could be accomplished (Steinberg *et al.*, "Rapid and Simple Single Nanogram Detection of Glycoproteins in Polyacrylamide Gels and on Electroblots," *Proteomics 1*:841-855 (2001)). A preferred glycoprotein stain is Pro-Q™ Emerald dye, Pro-Q™ Fuchsia dye or any other dye disclosed in US Serial no. 09/970,215.

Thus, it is particularly advantageous that the parallel determination of both protein expression levels and functional attributes of the proteins such as phosphorylation of proteins can be achieved with the present invention within a single 2-D gel electrophoresis experiment. Analysis can be accomplished by using image analysis software, e.g., Compugen's Z3 program. Any two images can be re-displayed, allowing visual inspection of the differences between the images, and quantitative information can be readily retrieved in tabular form with differential expression data calculated.

A particular advantage for staining phosphorylated proteins or peptides separated in polyacrylamide gels is for the analysis of proteins of interest as well as combining with mass spectrometry techniques for further analysis. For example, because phosphoproteins may co-migrate in a gel, further analysis may be desired to specifically identify and analyze the

phosphoprotein of interest. This further analysis can be achieved by measurement of a set of peptide masses derived from a protein, i.e., by peptide mapping with mass spectrometry (MS), or by obtaining amino acid sequence information from individual peptides, i.e., protein sequencing by MS/MS or by Edman degradation. Thus, a protein band or spot, once identified by the compositions and methods of the present invention, may be excised from the gel, rinsed, optionally reduced and S-alkylated, and then digested *in situ* in the gel with a sequence specific protease, e.g., trypsin. *See* Shevchenko *et al.*, "Mass Spectrometric Sequencing of Proteins from Silver Stained Polyacrylamide Gels," *Anal. Chem. 68*:850-58 (1996). The generated peptide mixture may be extracted from the gel and analyzed by MS. Peptide mapping by matrix assisted laser desorption/ionization (MALDI) mass spectrometry is often most sensitive. Methods for the in gel digestion of proteins are described in Jensen *et al.*, "Mass Spectrometric Identification and Microcharacterization of Proteins From Electrophoretic Gels: Strategies and Applications," *PROTEINS: Structure, Function, and Genetics Suppl. 2*:74-89 (1998).

Phosphorylated biomolecules immobilized on an array can also be detected using the methods and compositions of the present invention (Example 15). The array is incubated with a staining mixture for sufficient time to form a tertiary complex between a (un)labeled BAPTA compound, gallium ion and phosphorylated biomolecule. If the signal is not directly detectable, a fluorophore, an enzyme or hapten labeled anti-BAPTA or anti fluorophore antibody may be used to amplify the signal. Alternatively, the array may comprise BAPTA compounds complexed with gallium ions immobilized on the surface of the array wherein a sample thought to contain phosphorylated biomolecules is incubated with the array and detection of phosphorylated biomolecules occurs when the biomolecules bind the gallium/BAPTA complex and are illuminated. In this way, the array is used to detect and isolate phosphorylated biomolecules present as soluble molecules in solution. The present invention is thus useful, for example, as an array of protein substrates for various protein kinases (e.g., myosin light chain, MARCKS, myelin basic protein, casein, src-supressed C kinase substrate, insulin Receptor Substrate 1, Nuclear factor 90, Rap1, transcription factor stat5a). A sample is incubated with the array, if the detectable label is a fluorophore, for example, the coordinates of the fluorescent signals would provide a read-out of the kinases present in the fluid. Detection of phosphorylated biomolecules with an array offers many possibilities and the above description is not meant to limit how the present invention may be used in combination with array technology.

In addition, the methods and materials of the present invention are also useful for studying functional proteomics involving ligand overlay methodology. For example, arrayed proteins would be detected after incubation with phosphatidylinositol 4,5-bisphosphate (PIP2) micelles, followed by incubation with the staining mixture. The differences in staining would highlight an important class of phosphatidylinositide-binding proteins. Proteins such as SWI/SNF-like BAF, a chromatin remodeling complex and cofilin/ADF, a ubiquitous actin-binding protein, are likely to be identified using the methods of the present invention (Rando et al. PNAS (2202) 99(5):2824-9; Ojala et al. Biochem (2001) 40(51):15562-9). Another example of a ligand overlay assay would be GTP-binding proteins wherein the small GTP-binding proteins would be separated by high-resolution 2-D gel electrophoresis and subsequently transferred under renaturing conditions to a nitrocellulose membrane and probed with GTP. The bound GTP would then be stained with the staining mixture of the present invention resulting in identification of GTP binding proteins. A variety of other blot overlay nucleotide binding assays could be preformed using the staining mixture of the present invention wherein potentially any ligand and binding protein, wherein at least one of the pair contains phosphate group(s), could be used to identify novel binding proteins (Gromov et al. Electrophoresis (1994) 3-4:478-81).

Staining and isolation in a solution

In contrast, the compositions and methods of the present invention are also useful for staining, detecting and isolation of phosphorylated biomolecules that are not immobilized but free in a solution. A sample suspected of containing phosphorylated biomolecules is incubated with the staining mixture comprising fluorescent dye labeled BAPTA compounds wherein phosphorylated biomolecules are detected by fluorescence polarization (Example 14). Fluorescence polarization is based upon the finding that the emission of light by a fluorophore may be depolarized by rotational diffusion, or the rate at which a molecule tumbles in solution (*J. Phys. Rad. 1*:390-401 (1926)). Polarization is the measurement of the average angular displacement of the fluorophore, which occurs between the absorption and subsequent emission of a photon. This angular displacement of the fluorophore is, in turn, dependent upon the rate and extent of rotational diffusion during the lifetime of the excited state, which is influenced by the viscosity of the solution and the size and shape of the diffusing fluorescent species. If viscosity and temperature are held constant, for example, then polarization is directly related to the molecular volume or size of the fluorophore. Thus,

when detecting phosphorylated biomolecules in solution, the compounds and methods of the present invention contemplates taking advantage of fluorescent polarization, as described in U.S. Pat. No. 6,207,397. The detection of phosphorylated biomolecules would be based upon the observation that changes in polarization occur when a fluorescent dye labeled BAPTA compound undergoes a molecular weight change due to the binding of a phosphorylated biomolecule, for example, a phosphoprotein. The solution containing the sample and staining mixture are irradiated with plane-polarized light of a wavelength that is sufficient to excite the fluorophore. The light subsequently emitted by the fluorescent phosphorylated biomolecule is polarized to varying degrees depending on the molecular size of the fluorescent dye. In the unbound state in solution, low molecular weight labeled BAPTA compounds will rotate rapidly, and give low polarization readings. The degree of polarization of the emission can be measured without the necessity to separate the components in the solution.

Alternatively, phosphorylated biomolecules can also be isolated from a complex solution by taking advantage of the insoluble nature of the tertiary complex in an acidic environment. (Un)labeled BAPTA compounds, when part of the tertiary complex are insoluble at an acidic pH, thus when a staining mixture comprising BAPTA compounds is incubated with a sample in a way to facilitate formation of the tertiary complex, the complex will precipitate out of solution (Example 13). Therefore, typically the staining mixture and sample solution is vortexed, or mixed in a manner well known to those skilled in the art, to simultaneously facilitate binding and prevent aggregation of the tertiary complexes. Following formation of the tertiary complex the solution is treated in such a way as to isolate the precipitated complexes wherein a preferred method is centrifugation. The resulting pellet comprises phosphorylated biomolecules that can be further analyzed, by methods such as MS. This method takes advantage of the affinity "pull-down" of phosphopeptides or phosphoproteins from a complex solution (e.g., a cell extract protein digest) whereby at an acidic pH BAPTA compounds can complex with gallium ions and the phosphopeptides or phosphoproteins to form a precipitate. In addition, for the methods used to precipitate phosphorylated biomolecules from solution, ferric chloride comprising iron ions are also used for the formation of the tertiary complex.

Optionally, the present invention also contemplates the further isolation of the phosphorylated biomolecules wherein the labeled BAPTA compounds are removed from the

phosphorylated compounds resulting in a solution free of BAPTA compounds. This is accomplished when the labeled BAPTA compound optionally comprises a tag label such as a hapten wherein the tag label functions as a handle by which the BAPTA compound can be pulled away from the phosphorylated biomolecules. A preferred tag label is biotin wherein a matrix comprising biotin-binding proteins would be used as the medium to separate the BAPTA compounds from the phosphorylated biomolecules. Specifically, the resulting precipitation pellet is resuspended in a solvent that disassociates the gallium ion, BAPTA and phosphorylated biomolecules complex, such as a basic buffer, about pH 7-10. The solution is then added to the matrix, such as a column containing sepharose beads bound to biotin-binding protein, wherein the BAPTA-biotin compounds bind to the beads and the phosphorylated biomolecules pass through the column. The resulting eluent contains phosphorylated biomolecules free from BAPTA compounds that may be desirable for certain applications. Alternatively, the disassociated mixture of BAPTA-biotin and phosphorylated biomolecules can be incubated with beads comprising biotin-binding proteins as a slurry wherein removal of the beads by gravity, such as by size exclusion or centrifugation, results in a solution of phosphorylated biomolecules without BAPTA compounds. Preferred compounds for the formation of a precipitable tertiary complex are compounds 2, 5, 9 and 12.

The isolated phosphorylated biomolecules can be analyzed by a number of methods, including but not limited to, gel electrophoresis, MALDI-TOS MS, or LC-MS/MS. Additionally, as described below, the phosphopeptides can be derivatized using beta-elimination with subsequent addition of nucleophiles to aid in the identification the site of phosphorylation.

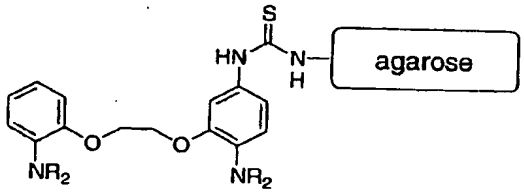
Isolation on a column

In addition to isolation of phosphorylated biomolecules from a complex sample in solution the present invention also contemplates the isolation of biomolecules using an affinity matrix (Example 15). Unlike in the precipitation method where an affinity column can be used to remove labeled BAPTA compounds from the isolated phosphorylated compounds, the affinity column in this method comprises unlabeled BAPTA compounds, which are subsequently bound with gallium ions following the addition of a gallium salt. A sample in an acidic binding buffer is then added to the mixture where present phosphorylated biomolecules will bind the gallium ions complexed to the BAPTA compounds. Isolation of the phosphorylated biomolecules is accomplished by an addition of a solution, which

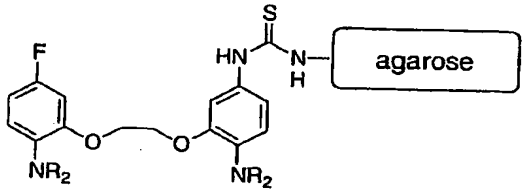
disassociates the tertiary complex of BAPTA, gallium ion and phosphorylated biomolecules. Preferably the elution solution would comprise a base and a basic pH buffering agent, useful bases include, without limitation, barium hydroxide, sodium hydroxide and ammonia hydroxide. Alternatively, basic amine solutions are also useful elution agents. Any base that is compatible with the sample and gallium ion phosphorylated biomolecule complex which disassociates the complex is preferred. In addition, organic solvents such as acetonitrile and alcohols are useful in eluting phosphorylated biomolecules from the BAPTA gallium affinity column, and may be preferable depending on the subsequent analysis of the phosphorylated biomolecules, such as with MS. The affinity column matrix comprises BAPTA compounds bound to agarose (Compound 13 and 14) or a resin; preferably the matrix comprises BAPTA-agarose in a column (immobilized affinity column (IMAC)). The acidic binding buffer uses the same components as the staining mixture, as described in detail above.

Preferred BAPTA compounds for use with an immobilized affinity column are:

Compound 13, wherein R is CH_2CO_2.



Compound 14, wherein R is CH_2CO_2 and the BAPTA component is fluorinated at the R^3 position.



As many phosphorylated biomolecules often exist only in small abundances, the column of the present invention is especially useful for the purification and enrichment of such phosphorylated biomolecules. These columns are useful for purifying phosphorylated

peptides from crude peptide mixtures, which is advantageous for methods analyzing the peptides by matrix assisted laser desorption/ionization (MALDI) MS or nanoelectrospray tandem mass spectrometry (MS/MS). It is contemplated that a wide variety of methods may be used to prepare samples purified and/or enriched by the gallium IMAC column or separated from a complex solution. For example, dried separated phosphopeptides may be resuspended in water for LC-MS analysis.

The IMAC of the present invention is also readily adaptable to microfluidics such as the CD technology developed by Gyros AB (Uppsula, Sweden) wherein high throughput screening of samples for proteomic analysis, such as peptide mapping with MALDI-TOF, can be accomplished. Briefly, the Gyros AB technology comprises a CD microlaboratory with hundreds of microstructures (columns) wherein samples are run through the columns based on centrifugation speeds and the eluted sample is analyzed on the CD, allowing for the entire process from a protein digest to MS analysis on the CD. The columns can be packed with particles comprising BAPTA compounds (Compound 13 or 14), samples are run through the columns and either analyzed for phosphorylated peptide concentration, by fluorescent signal, or applied to a matrix on the CD for MALDI-TOF analysis. Thus, the methods of the present invention are amenable to microfluidics for high throughput screening of samples, which is advantageous for proteomic studies.

Thus, the invention provides analytical reagents for use with mass spectrometry-based methods for the rapid, and quantitative analysis of phosphoproteins or phosphopeptides in a mixture. The reagents and methods can be applied to the detection and identification of proteins in sample mixtures of proteins, where the peptides isolated by the method are characteristic of the presence of a protein in the mixture. Isolated peptides or proteins may be characterized by mass spectrometric (MS) techniques, and by application of sequence database searching techniques for identifying the protein from which the sequenced peptide originates.

The following references are examples of mass spectrometric techniques for protein identification, and can be used with the materials and methods of the present invention: Ideker *et al.*, "Integrated Genomic and Proteomic Analyses of a Systematically Perturbed Metabolic Network," *Science 292*:929-934 (2001); Gygi & Aebersold, "Measuring Gene Expression by Quantitative Proteome Analysis," *Curr. Opin. Biotechnol. 11*:396-401 (2000);

Goodlett *et al.*, "Protein Identification with a Single Accurate Mass of a Cysteine-containing Peptide and Constrained Database Searching," *Anal. Chem. 72*:1112-8 (2000); Goodlett *et al.*, "Quantitative In Vitro Kinase Reaction as a Guide for Phosphoprotein Analysis by Mass Spectrometry," *Rapid. Commun. Mass. Spectrom. 14*:344-8 (2000); *McLachlin & Chait*, "Analysis of Phosphorylated Proteins and Peptides by Mass Spectrometry," *Current Opin. Chem. Biol. 5*:591-602 (2001); Aebersold & Goodlett, "Mass Spectrometry in Proteomics," *Chem. Rev. 101*:269-295 (2001); Vener *et al.*, "Mass Spectrometric Resolution of Reversible Protein Phosphorylation in Photosynthetic Membranes of *Arabidopsis thaliana*," *J. Biol. Chem. 276*:6959-66 (2001); Zhou *et al.*, *Nature Biotechnol. 19*:375-8 (2001). Those of skill in the art will recognize these currently available mass spectrometry methods as compatible with the materials and methods of the present invention. However, the present invention also contemplates that the materials and methods can be used with mass spectrometry techniques yet to become available that achieve the same results. In addition, it is contemplated by the invention that prior to detection, phosphopeptides may be subjected to Reverse Phase or ion exchange columns to remove undesired materials from the phosphopeptide sample.

The present invention also contemplates alternative methods of purification and/or enrichment. For example, the materials and methods of the present invention may be used with Luminex technology, which involves the labeling of dyes on latex microbeads with two fluorophores. Using precise ratios of the two fluorophores, a hundred different bead sets may be created, each one being unique and distinguishable in a laser beam, based on the color code that results form the ratio of the two dyes. Instead of a capture antibody for a specific molecule coupled to a specific bead set, the metal chelators of the present invention may be used. For example, after an analyte is bound to the gallium and metal chelator complex on the bead, a detector antibody coupled to phycoerythrin may be used as a reporter. The end result is an antibody/metal chelator sandwich assay on the color-coded microbead. The beads and the reporter molecule may be read on a Luminex 100 instrument using a dual laser system as they pass through a flow cell. One laser detects the beads (the color code for an assay) and the other laser detects the reporter signals. Thus, it is contemplated by the present invention that instead of a detector antibody, the metal chelating complex may be used in accordance with the bead detection for the separation and detection of phosphorylated biomolecules.

In the alternative, magnetic bead separation for automated bead and particle capture systems, for example, LifeSep magnetic beads by Dexter Magnetic Technologies, may be used with the materials and methods of the present invention. Magnetic separation works by means of specific affinity coatings attached to tiny magnetic beads. Beads are mixed with a sample containing phosphorylated biomolecules such that the phosphorylated biomolecules have the opportunity to bind tightly to the gallium ion/BAPTA complex on the bead. Once attached, the bead and the tertiary complex can be separated using a strong magnetic field. Depending on the process, the phosphorylated biomolecule may either be left bound to the bead or released by washing in a suitable solvent or a basic buffer. Thus, efficient and rapid isolation is therefore possible and therefore, it is contemplated by the present invention, that the BAPTA/gallium ion complex may be used with well-known methods of magnetic bead separation.

Thus, a wide variety of materials and methods are provided for the separation, purification and enrichment of phosphorylated biomolecules, including the use of novel immobilized affinity column (IMAC).

Identification of the Sites of Phosphorylation in Proteins and Peptides

The present invention provides compounds and methods for the differential isolation and identification of phosphorylated serine, threonine or tyrosine amino acids. The materials and methods described above for the staining and isolation of phosphorylated biomolecules, absent mass spectrometry or other similar techniques, are generally used for detecting protein phosphorylation, but do not give information on the specific location and quantitation of the phosphate on the protein or polypeptide. The present invention comprises further analyzing isolated phosphorylated proteins or peptides obtained by IMAC or precipitation methods described above to differently identify phosphorylated peptides. Isolated phosphorylated proteins are subjected to proteolytic digestion followed by acid hydrolysis or alkaline hydrolysis and analyzed.

A base such as barium hydroxide or sodium hydroxide catalyzes the dephosphorylation of the peptides forming activated dehydroalanine derivatives, which are vulnerable to attack by amine or thiol containing compounds resulting in the formation of stable phosphopeptide derivatives. These derivatives are more hydrophobic and are therefore more amenable to identification by HPLC, mass spectrometry, or by Edman sequencing. Under the conditions

used, phosphoserine residues undergo elimination and addition, phosphothreonine residues undergo elimination but not addition and phosphotyrosine residues are unaltered by the treatment. Thus, differential identification can be accomplished based on this knowledge. In Edman degradation, during the acid or base delivery, the phosphate is β-eliminated and the resulting dehydro-amino acids rapidly form a dithiothreitol (DTT) adduct. *See* Meyer *et al.*, FASEB J. 7:776 (1993). In contrast, O-HexNAc on serine and threonine is stable in Edman degradation. *See* Gooley & Williams, *Nature 358*:557 (1997). Thus, the present invention may be used to differentiate between serine or threonine phosphorylation and glycosylation.

Edman degradation is thus an effective method for quantitating serine and threonine following β-elimination and derivatization. See Yan *et al.*, "Protein Phosphorylation: Technologies for the Identification of Phosphoamino Acids", *J. Chrom.* 808:23-41 (1998)). These modified products also survive acid hydrolysis, and can be quantitated by reversed-phase HPLC analysis. *See, e.g.*, Meyer *et al.*, *J. Chromatogr. 397*:113 (1987) and Holmes, *FEBS Lett. 215*:21 (1987). Using a similar approach, characterization by capillary zone electrophoresis and laser-induced fluorescence has also been used to quantitate the serine content of peptides and proteins. See Fadden & Haystead, Anal. Biochem. 225:81 (1995).

Nanoelectrospray MS/MS is used for phosphopeptide sequencing for exact determination of phosphorylation sites. *See* Stensballe *et al.*, "Characterization of Phosphoproteins From Electrophoretic Gels by Nanoscale Fe(III) Affinity Chromatography With Off-Linc Mass Spectrometry Analysis," *Proteomics 1*:207-222 (2001). In-gel digestions can be achieved as described in Shevchenko *et al.*, *Anal. Chem. 68*:850-58 (1996) and Jensen *et al.*, *Meth. Mol. Biol. 112*:513-30 (1998). The present invention also contemplates that the materials and methods can be used with mass spectrometry techniques yet to become available that achieve the same results.

The sequence of phosphopeptide and the identification of the site(s) of phosphorylation can also be determined by a combination of tandem mass spectrometry and computer-assisted database search programs, such as SEQUEST (Trademark, University of Washington, Seattle WA) (McCormack *et al.*, "Direct Analysis and Identification of Proteins in Mixtures by LC/MS/1\4S and Database Searching at the Low-Femtomole Level," *Anal. Chem. 69*:767-776 (1996); Eng *et al.*, "An Approach to Correlate Tandem Mass Spectral Data of Peptides

with Amino Acid Sequences in a Protein Database," *J. Amer. Soc. Mass. Spectrom.* 5:976-989 (1994); U.S. Patent No. 5,538,897. While a variety of MS methods are available and may be used in these methods, Matrix Assisted Laser Desorption Ionization MS (MALDI/MS) and Electrospray Ionization MS (ESI/MS) methods are typically used.

Measuring Kinase and Phosphatase Activity

The compositions and methods of the invention find use in quantifying the activity of a selected protein kinase or phosphatase using a variety of substrates. Current commercial kinase assays are often time-consuming and require many steps such as electrophoresis, centrifugation, ELISA or immunoprecipitation. The present invention provides methods for the rapid, sensitive, and non-radioactive detection of a variety of selected kinases and phosphatases and provides, in addition, methods that are well suited for high-throughput screening. The kinase and phosphatase assays of the present invention will also permit the screening of inhibitors and activators of, for example, tyrosine kinases and, in addition, will also permit the monitoring and the purification of kinase and phosphatase enzymes.

Specifically, a kinase substrate is covalently or non-covalently attached to a surface such as a microwell plate and the assay is preformed in a non-continuous heterogeneous manner. The kinase substrate comprises a kinase consensus phosphorylation site, preferably a peptide or a random polymer (poly(Glu:Tyr), poly(Glu:Ala:Tyr). Optionally the kinase substrate comprises a fluorophore. A sample suspected of containing a kinase is combined with the kinase substrate along with ATP wherein an active kinase enzyme will add phosphates to the kinase substrate. The addition of phosphate groups is measured after removal of the kinase solution and adequate washing wherein a staining mixture, as described above, is added to the kinase substrate. Typically the BAPTA comprises a fluorophore and the kinase activity is measured by illuminating the fluorophore such as with a fluorescent plate reader. Alternatively the BAPTA comprises an enzyme such as peroxidase wherein the kinase activity would be measured after addition of the appropriate enzyme substrate and detection with a plate reader. In addition, using an inhibitor of the selected kinase or phosphatase in the assay, for example, by using sodium orthovanadate may enhance the specificity of the kinase. Alternatively the assay is easily adaptable to measure phosphatase activity wherein phosphatase substrate, phosphorylated peptides or proteins, would be bound to a solid matrix such as a microwell plate.

The materials and methods of the present invention may also be used to detect and/or quantitate kinases or phosphatases by employing a FRET-based assay. For example, a peptide labeled with a fluorophore may be combined with the BAPTA/gallium ion complex derivatized with phycoerythrin. When the peptide is phosphorylated, the peptide binds phycoerythrin and the emission maximum shifts in the assay. Time-resolved fluorescence may be achieved, for example, by employing an europium-based chelate on the peptide and the BAPTA/gallium ion complex derivatized with allophycocyanin. The donor fluorophore may be excited, in this example, at 335 nm and an emission shift from 620 nm to 665 nm indicates peptide interaction with the metal chelator and gallium complex.

Thus, in one aspect of the invention numerous enzymes, including nitrogenase, phosphoribosyl-pyrophosphate synthetase, undecaprenyl pyrophosphate synthase, DNA polymerases, RNA polymerases, farnesyltransferase, nucleoside triphosphate pyrophosphohydrolases, pyrophosphate-fuctose-6-phosphate 1-phosphotransferase (PFPPT), sulfate adenyltransferase, UTP-glucose-1-phosphate uridinyltransferase (UGPP), asparagines synthetase, and UDP-glucose pyrophosphorylase involve the metabolism of inorganic pyrophosphate and thus are potential targets for quantitation by the disclosed invention.

Illumination

At any time after or during staining, the sample is illuminated with a wavelength of light capable of exciting the reagent to produce a detectable optical response, as defined above, and observed with a means for detecting the optical response. Equipment that is useful for illuminating the dye compounds of the invention includes, but is not limited to, hand-held ultraviolet lamps, mercury arc lamps, xenon lamps, lasers and laser diodes. These illumination sources are optionally integrated into laser scanners, fluorescence microplate readers, standard or minifluorometers, or chromatographic detectors. The degree and/or location of staining, compared with a standard or expected response, indicates whether and to what degree the sample possesses a given characteristic, i.e. phosphorylated biomolecules.

The optical response is optionally detected by visual inspection, or by use of any of the following devices: CCD cameras, video cameras, photographic film, laser-scanning devices, fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, fluorescence microplate readers, or by means for amplifying the signal such as photomultiplier tubes.

The detectable optical response can be quantified and used to measure the degree of phosphorylation of the phosphorylated biomolecule in the sample mixture. Quantification is typically performed by comparison of the optical response to a prepared standard or to a calibration curve. Typically, the measured optical response is compared with that obtained from a standard dilution of a known concentration of the phosphorylated biomolecule in an electrophoretic gel, or on a membrane. Generally a standard curve must be prepared whenever an accurate measurement is desired. Alternatively, the standard curve is generated by comparison with a reference dye or dyed particle that has been standardized versus the reagent-target conjugate desired.

Alternatively, stained electrophoretic gels are used to analyze the composition of complex sample mixtures and additionally to determine the relative amount of a particular phosphorylated biomolecule in samples. This can be accomplished in conjunction with determination of the number of phosphate groups on a molecule and a total protein stain to differentiate between an increase in the amount of protein versus an increase in phosphate groups on a particular protein or peptide.

Thus, it is contemplated by the present invention that a wide variety of instrumentation may be used to detect the phosphorylated biomolecules, e.g., electrospray ionization (ESI) tandem mass spectrometry (MS/MS). A series of different techniques, including automated high performance liquid chromatography (HPLC)-MS/MS, capillary-HPLC-MS/MS, and solid phase extraction (SPE)-capillary zone electrophoresis (CZE)-MS/MS, are described in Figeys *et al.*, "Electrophoresis Combined With Novel Mass Spectrometry Techniques: Powerful Tools for the Analysis of Proteins and Proteomes," *Electrophoresis 19*:1811-1818 (1998), all of which are incorporated herein by reference.

When measuring fluorescence polarization, many forms of automation may be used and are known by those skilled in the art. As one example, any standard fluorometer equipped for polarization experiments or measurements may be used in practicing this embodiment of the invention to both irradiate the mixture and measure the polarization. Wavelengths suitable to excite the fluorophore depend on the nature of the fluorophore, as described above. Typically, one uses cut-off filters to define a wavelength range which is determined by the excitation and emission wavelengths of the fluorophore. For example, for fluorescein

carboxyamide peptides, one would typically use an excitation cutoff filter of 485nm. Standard fluorometers can be used, or, for example, a plate reader. Thus, in addition, one of skill in the art of automation may use various instruments to measure fluorescence polarization in accordance with the materials and methods of the invention.

As described above, while a wide variety of methods of detection are used with the present invention, preferred methods include the use of fluorescence. Fluorescence from the BAPTA gallium ion complex binding to the phosphorylated biomolecule can be visualized with a variety of imaging techniques, including ordinary light or fluorescence microscopy.

III. KITS OF THE INVENTION

Suitable kits for staining and isolating a phosphorylated biomolecule also form part of the invention. Such kits can be prepared from readily available materials and reagents and can come in a variety of embodiments. The contents of the kit will depend on the design of the assay protocol or reagent for detection or measurement. All kits will contain instructions, appropriate reagents and label, and solid supports, as needed. Typically, instructions include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be added together, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like to allow the user to carry out any one of the methods or preparations described above.

A kit for staining phosphorylated biomolecules comprises a staining mixture, a total protein stain, wherein the staining mixture is identical to what was described above. Optionally, the kit would comprise any one of the following; molecular weight markers for both phosphorylated and unphosphorylated biomolecules and staining solution for glycoproteins. When the kit is used to detect phosphorylated proteins or peptides in a gel or on a blot, molecular weight markers are typically part of the kit. Alternatively, when the kit is used to stain phosphorylated biomolecules in a solution or on an array molecular weight markers would typically not be part of the kit.

Another kit of the present invention finds use in isolating phosphorylated proteins or peptides from a complex sample mixture wherein the tertiary complex is precipitated out of solution. The kit would optionally comprise a staining mixture wherein the BAPTA compound is a

biotinylated labeled BAPTA, elution buffer and biotin binding support wherein the support could be a multi-well plate, agarose resin or magnetic beads such that a biotin-binding protein is covalently attached to the support. The kit would further optionally contain a spin column, standard peptide mixture and nucleophilic derivatization compound.

Another kit of the invention that finds use in isolating phosphorylated proteins or peptides from a complex sample mixture comprises a column containing BAPTA-agarose compounds. The kit would typically comprise, in addition to the column, a gallium salt, a wash buffer, an acidic binding buffer, and a basic eluting buffer wherein the gallium salt is preferably gallium chloride and the base is preferably barium hydroxide.

Those skilled in the art will appreciate that a wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit, and the particular needs of the user.

IV. APPLICATIONS

The present invention is useful for a wide variety of applications in a wide variety of areas including, but not limited to, basic research applications, high-throughput screening, proteomics, microarray technology, diagnostics, and medical therapeutics. Those skilled in the art will appreciate that the invention can be used in a wide variety of assay formats in a wide variety of diagnostic applications. The foregoing description seeks merely to illustrate the many applications of the materials and methods of the present invention, and does not seek to limit the metes and bounds of the invention as described in the above sections.

The materials and methods of the present invention are useful for a number of applications. The present invention may be used to generate data that is used as a reference point for a human patient or animal sample for the diagnosis of disease, progression of disease, and/or predisposition for disease. By way of example, if a disease is associated with changes in protein composition in certain cells, i.e. protein phosphorylation in different organ systems, cell sources or tissue types, a patient sample may be used to generate a protein profile according to the materials and methods of the invention, and compared with profiles of corresponding samples of normal or non-diseased samples and/or diseased origin to

determine the presence or absence of, progression of, and/or predisposition to the particular disease in question. It is contemplated by the present invention that many diseases may be diagnosed with data or images generated by the materials and methods of the present invention, including diseases for which particular aberration in protein expression are known. Such disease states include, but are not limited to, metabolic diseases that are associated with the lack of certain enzymes, proliferative diseases that are associated with aberrant expression of certain genes, e.g., oncogenes or tumor suppressors, or developmental diseases that are associated with aberrant gene expression. Thus, if it is known that a given disease of interest is associated with certain changes of a particular type of cell, tissue, cell source, or organ system, a human patient or animal may be diagnosed simply based on its individual expression profile generated by 2-D gel electrophoresis in accordance with the present invention. In another aspect, expression profiles generated by 2-D gel electrophoresis may be used to analyze a diseased organ, tissue or cell type and compared with the corresponding profile counterpart obtained from a non-diseased sample.

Moreover, the information generated by the materials and methods of the present invention may be used to "backtrack" or identify and/or associate novel or known genes and their corresponding products that are involved in the manifestation of, progression of, or predisposition to a disease of interest, and with the development of symptoms of a particular disease, by generating the amino acid sequence of a phosphoprotein or phosphopeptide of interest based on the materials and methods of the present invention. For example, ESTs are partial nucleotide sequences obtained from cDNA derived from mRNA from any given cell line. Thus, the present invention may be used to generate amino acid sequence data, and from the amino acid sequence data, extrapolate potential DNA sequences that can be used to search EST databases. For example, MS/MS sequence data in the form of a peptide sequence tag, may be used to query EST databases if a protein is not identified by searching the conventional full length sequence databases. If an EST is retrieved, then the corresponding DNA clone can be ordered and sequenced. The apoptotic protease FLICE/Caspase-8 and the trinucleotide repeat binding protein p20-CGGBP was recently identified and cloned by this approach. See Muzio *et al.* "FLICE, a Novel FADD-homologous ICE/CED-3-like Protease, is Recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex," *Cell 85*:817-827 (1996) and Deissler *et al.*, "Rapid Protein Sequencing by Tandem Mass Spectrometry and cDNA Cloning of p20-CGGBP. A Novel Protein that Binds to the Unstable Triplet Repeat 5'-d(CGG)n-3' in the Human FMR1 Gene," *J. Biol. Chem. 272*:16761-16768 (1997). During

analysis of protein components isolated from the human spliceosome a relatively large number of ESTs were retrieved by MS/MS data. In the alternative, it may be necessary to generate amino acid sequence data for sequence homology searching, e.g., by BLAST algorithm searching. If the sequence is significantly related to a characterized protein from another species, then its function may be directly deduced. If no related proteins exist, however, then the amino acid sequence data may be used to design oligonucleotide probes for cloning of the cognant gene. Complete sequence determination of the protein can then be performed at the DNA level by established genetic and molecular biology techniques.

The sources of phosphorylated biomolecules can be from many different sources, including cell types, cell conditions, genetic background, states of perturbation or of different developmental states. Cell sources for analysis may be; transgenic or nontransgenic, transfected or nontransfected, virus-or prion-infected or noninfected. "Perturbation" refers to experimental manipulation of the sources, i.e. cells, such as treatment with a particular compound or drug compared to non-treatment of a drug. Alternatively, it can refer to treatment with a particular compound or drug compared to treatment of a source or cell with a different dosage of a particular compound or drug.

For example, cells can be subjected to a candidate drug regimen to generate a phosphoprotein expression profile in accordance with the present invention. The images of 2-D gels generated in accordance with the present invention may be stored on a database, and the database may be later queried for a cell source representing a different treatment, e.g., protein expression profiles generated by a response to a different drug or where no drug is present, or where the candidate drug is used in a different way. Moreover, the candidate drug may bind specifically to a particular protein, permitting analysis of cells or other sources, which express that protein. The database query may derive information about cell sources that express a particular protein.

Thus, the materials and methods of the present invention could be used to gain valuable information of the effects of various drugs and compounds on the cellular phosphoprotein status. For example, it was recently demonstrated that the compounds FK-506, cyclosporin and rapamycin, used to suppress tissue rejection, inhibit certain protein phosphatases. Schreiber *et al.*, *Cell 70*:365-68 (1992). A database of lymphoid proteins detected by two-dimensional polyacrylamide gel electrophoresis has also been generated. The database

contains 2-D patterns and derived information pertaining to polypeptide constituents of unstimulated and stimulated mature T cells and immature thymocytes, cultured T cells and cell lines that have been manipulated by transfection with a variety of constructs or by treatment with specific agents, single cell-derived T and B cell clones, cells obtained from patients with lympoproliferative disorders and leukemia, and a variety of other relevant cell populations. *See* Hanash & Teichroew, "Mining the Human Proteome: Experience with the Human Lymphoid Protein Database," *Electrophoresis 19*:2004-2009 (1998). Thus, in accordance with the present invention, cells treated with a suspected drug compound can be compared to untreated cells to generate a 2-D gel electrophoresis profile. Furthermore, it may be observed, for example, that certain drug compounds induce the activation of different sets of kinases or phosphatases. Such evidence could lead to the elucidation of the mechanism by which many drug compounds work and manifest their effects.

A 2-D gel electrophoresis study was performed to generate a phosphoprotein profile in cultures that were subjected to the effect of oxygen/glucose deprivation. The results suggested that this model could be a good method to observe the development of the tissue and its response to an ischaemic lesion. See Tavares *et al.*, "Profile of Phosphoprotein Labeling in Organotypic Slice Cultures of Rat Hippocampus," *Neurochemistry 12*:2705-2709 (2001).

The materials and methods of the present invention can also be used to study biological phenomena, such as, for example, signal transduction, mitosis, cell proliferation, cell motility, cell shape, gene regulation, and many other cellular processes. The mechanism of action of kinases and phosphatases and the physiological relevance of site-specific phosphorylation of substrate proteins can be explored with the materials and methods of the present invention. The materials and methods of the present invention offer the advantage of high-resolution 2-D gel electrophoresis to simultaneously resolve hundreds of cellular polypeptides. Using the materials and methods of the present invention, the potential for the identification of proteins and the expression of their genes at various stages of cell growth, differentiation, or disease, is extensive. Thus, the invention provides methods and materials for the detection and quantitation of phosphorylation of specific cellular proteins that may provide insight into the mechanisms by which phosphorylation is employed for the regulation in cells.

It is well known that the critical events in the cell cycle are controlled by a complex interplay of kinases and phosphatases. Thus, the status of phosphorylation of different protein isoforms during different phases of the life cycle is important to researchers. Thus, in accordance with the materials and methods of the present invention, the phosphorylation of different proteins related to the stage of the cell cycle related to the activity of certain kinases or phosphatases may be explored using the materials and methods of the present invention. By way of example, a global analysis of phosphoproteins in cells can be used to analyze the primary signals of, for example, mitogenesis in selected cells, or in G1 or S phase cells. Thus, the materials and methods of the present invention may be useful in investigating the phosphorylation status of various proteins during the cell cycle.

Those of skill in the art will recognize that a database can be generated using the materials and methods of the present invention to produce a record that may show the correlation between gene expression at the RNA and protein level to the function of the cell. For examples, in situations where the cells under study are obtained in both cancerous and normal conditions, comparison of the relative gene expression can be used to identify genes which can serve either as diagnostic markers of pathology or as sites for the pharmacological intervention or treatment of, for example, cancer. Similarly, other diseases can be analyzed merely by substituting the source of cells for analysis.

Thus, the present invention may be used to generate a comprehensive phosphoprotein expression profile from any cell type or biological fluid of interest. A cell type of interest may be any cell, or portion thereof with genetic material. A reference cell can be any cell type the difference in protein expression patterns and levels is desired to be measured against. Preferably the cells are maintained as similar to their native state as possible and culture techniques, incubation times etc. are performed identically between the two to minimize any non-naturally occurring differences. For example, development of a comprehensive protein profile of pre-cancerous, and/or malignant test cells and a normal reference cell can be achieved according to the invention. Such expression profiles can be used to characterize molecular events, for example, related to tumor development and the cellular mechanisms involved.

In accordance with the present invention, a cell of interest and a reference cell could be obtained from the same patient to get an individual phosphoprotein expression profile that

can be used to diagnose or treat that patient for those diseases that involve protein phosphorylation. For example, when a tumor is excised, a margin of non-transformed cells is typically removed as well. Phosphoprotein expression profiles can help to ensure that the cells removed all had similar profiles to normal cells rather than the metastatic cells from the same patient for those cancers that involve, or are thought to involve, protein phosphorylation.

One example of cell lines that may be analyzed using the materials and methods of the present invention include human tumor cell lines. For example human tumor cell lines representing a broad spectrum of human tumors and exhibiting acceptable properties and growth characteristics may be grown according to standard methods for cell line expansion, cryopreservation and/or characterization for use with the present invention. If phosphorylation is implicated in cellular aging, the materials and methods of the present invention may be used to analyze test and reference cells, i.e., to develop phosphoprotein expression profiles associated with aging, such as different stages of ontogenesis, for example, protein profiles of embryonic liver-derived hematopoietic stem cells. Thus, the invention contemplates a comparison of any diseased state to a normal reference state.

In addition, studying the effects of various ligands added to cells can assess the effects of various agonists on the reversible phosphorylation on multiple cellular proteins. Thus, for example, the *in vivo* substrates of a kinase of interest could be determined by treating cells with suspected substrates and comparing the resulting gel images of 2-D separated proteins with untreated controls. As an increasing number of cytokines are being discovered and characterized, many or all of which will activate protein kinase or phosphatases as they manifest their effects on target cells, the materials and methods of the present invention may be especially useful for exploring such mechanisms. For example, the identity of some of these proteins may suggest assays to be formulated for the location and characterization of kinases and phosphatases induced by lymphokines or cytokines. Methods for identifying phosphoproteins upregulated in response to the cytokines IL2 or IFNα were described using both silver staining and Western blotting for protein detection and identification. The silver-stained profile served as a "fingerprint" for phosphorylation events that occur in response to cytokine treatment. *See* Stancato & Petricoin III, "Fingerprinting of Signal Transduction

Pathways Using a Combination of Anti-Phosphotyrosine Immunoprecipitations and Two-Dimensional Polyacrylamide Gel Electrophoresis," *Electrophoresis* 22:2120-2124 (2001).

The materials and methods of the present invention can also be used to map kinase and phosphatase substrates *in vitro*. For example the identification of substrates for various kinases can be determined by processing extracts from cells and allowing a purified kinase to phosphorylate its substrate proteins. One skilled in the art could compare all the cytosolic proteins as candidate substrates for the kinase under investigation to identify major substrates for a kinase of interest. Similar to *in vitro* assays for kinases, it is possible to use the advantages offered by 2-D separation and the invention to isolate and characterize the phosphatases that catalyze the removal of phosphate from phosphorylated substrates. Thus, the activity of kinases and phosphatases responsible for phosphorylating and dephosphorylating individual proteins can be analyzed. *See, e.g.*, Fruehling & Longnecker, "In Vitro Assays for the Detection of Protein Tyrosine Phosphorylation and Protein Tyrosine Kinase Activities," *Methods in Mol. Biol. 174(Ch. 36)*:337-343 (2001).

The applications described herein are provided merely to illustrate a wide variety of potential uses of the invention, and are in no way intended to limit the scope of the invention. A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the invention and shall not be construed as being a limitation on the scope of the invention or claims.

EXAMPLES

Generally, the nomenclature as used herein, and the laboratory procedures in cell culture, molecular genetics, and protein chemistry described below are those well known and commonly employed in the art. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. Units, prefixes, and symbols may be denoted in their SI accepted form. Numeric ranges are inclusive of the number defining the range and include each integer within the defined range.

Example 1: Determination of BAPTA specificity for gallium and gallium ions for phosphorylated biomolecules

(A) BAPTA with trivalent gallium ions specifically detects phosphoproteins.

A comprehensive search of metal chelating compounds was performed to identify fluorescent reagents that when combined with gallium chloride would specifically detect phosphoproteins. The compounds were tested in a fluorescence spectrophotometer for their ability to bind gallium (III) ion and specifically detect the phosphoprotein ovalbumin. Binding to gallium (III) ion was determined by a fluorescence increase of the compound in the presence of up to 5 μM gallium chloride in 75 mM Sodium Acetate (pH 4.0) and 140 mM NaCl. Ovalbumin detection was also judged by a fluorescence increase, however the compounds were placed in a solution containing, 75 mM Sodium Acetate (pH 4.0), 140 mM NaCl, 1-4 μM ovalbumin, and 0.5 μM gallium chloride. Specificity of phosphoprotein detection was evaluated by elimination of the fluorescence increase in the presence of the same solution lacking gallium chloride.

Using compound 1 a variety of metal ions, including iron and gallium, were screened to determine which ion(s) were best suited for phosphoprotein detection. Metal ions were assayed for binding to compound 1, phosphoprotein detection, and general protein staining by monitoring a fluorescence increase at 530 nm in 75 mM Sodium Acetate (pH 4.0), 140 mM NaCl, 0.5-5 μM metal ion, with or without 4 μM ovalbumin or 1 μM lysozyme. Only trivalent cations bound to compound 1 and resulted in a fluorescence increase at 530 nm and only gallium (III) ion was capable of specifically indicating phosphoproteins when bound to compound 1. Therefore, gallium (III) ion is the most preferred metal ion for phosphoprotein detection.

(B) Differential binding affinity of compound 1 for phosphate compounds.

Compound 1 complexed with gallium (III) ion has differential affinities for various phosphate substrates in 75 mM Sodium Acetate (pH 4.0) and 140 mM NaCl. Some of the phosphate-containing compounds studied were inorganic phosphate, phosphate attached to a protein, a peptide or an amino acid, pyrophosphate, ATP, and DNA. The affinities for these phosphate substrates for the BAPTA gallium (III) ion reagent were ~ 50 μM for inorganic phosphate and phosphate attached to a protein, a peptide or an amino acid, ~ 200 nM for pyrophosphate and ATP, and no binding was detected for DNA. Compare these values to the affinity of compound 1 for gallium (III) ion of 2.5 μM. Most known phosphate compounds should fall into one of these three categories with respect to how it will bind to BAPTA: gallium (III) ion; 1) single phosphate group (i.e. inorganic phosphate or phosphate on a protein), 2)

multiple linked phosphate group (i.e. pyrophosphate or ATP), or 3) bridging phosphate group (i.e. nucleic acids).

(C) Compound 4 displays dual-emission wavelengths upon binding to gallium (III) ion and phosphate.

Concentrations of 0.1-1.0 μM of compound 4 in a solution of 75 mM Sodium Acetate (pH 4.0) and 140 mM NaCl display a single emission peak centered at 410 nm (excitation 350 nm). Addition of 10 nM to 1 mM gallium chloride results in a decrease in 410 nm emission and a concomitant increase in emission at 490 nm with an isosbestic point of 475 nm. The half maximal response for this transition from the blue to green emitting state occurs at approximately 1.8 μM gallium chloride. Therefore, 0.1 μM compound 4 with 1.7 μM gallium chloride in 75 mM Sodium Acetate (pH 4.0) and 140 mM NaCl display both the 410 nm and 490 nm emission peaks. The addition of phosphate can alter the equilibrium between the emission peaks in favor of the longer wavelength 490 nm peak.

Example 2: Detection of Phosphoproteins in SDS-Polyacrylamide Gels

Phosphoproteins were separated by SDS-polyacrylamide gel electrophoresis utilizing a 4% T, 2.6 % C stacking gel, pH 6.8 and 13% T, 2.6% C separating gel, and pH 8.8, according to standard procedures. % T is the total monomer concentration (acrylamide + crosslinker) expressed in grams per 100 ml and % C is the percentage crosslinker (e.g. N, N'-methylene-bis-acrylamide, N,N'-diacryloylpiperazine or other suitable agent). The separating gels were 8 cm wide by 5 cm high and 0.75 cm in thickness. After electrophoresis, the gels were fixed by immersing them in 100 ml 45% methanol, and 5% acetic acid for 90 minutes. The gels were washed twice in water for a total of 30 minutes. The gels were then added to a staining solution and incubated for 120 minutes at room temperature with gentle orbital shaking, typically 50 rpm. The staining solution contained 50 mM sodium acetate (pH 4.0), 250 mM sodium chloride, 20% v/v 1, 2-propanediol, 1 μM compound 2, 1 μM gallium chloride. 120 μl of compound 2, in stock solution at 1mM, and 120 μl of gallium chloride, in stock solution at 1 mM, were added to 1080 μL water. This mixture was then added to 59 ml of the staining buffer. Alternatively the labeled BAPTA and the gallium chloride may be added separately, directly to 60 ml of the staining diluent. After incubation in staining solution, the gel was

washed with 75 ml 50 mM sodium acetate (pH 4.0), and subjected to 2 washes of 30 minutes each.

Images were acquired on a Fujifilm FLA 3000 laser scanner using 532 nm excitation and 580 nm bandpass emission filters. The data was displayed using Image Gauge Analysis software. Images of phosphoproteins were displayed as dark bands. Proteins not containing phosphate were not stained or were very lightly stained relative to the phosphoproteins. When gels were stained as above but with gallium chloride omitted from the staining solution, phosphoproteins were not selectively stained, and could not be distinguished from background or light nonspecific staining. Gels were washed overnight with 50 mM sodium acetate (pH 4.0) and images were acquired as above. The background and nonspecific staining was further reduced relative to phosphoprotein staining.

Fixation in methanol/acetic acid may be done overnight or the gels may be left in fixative for several days. Other salts may be used instead of sodium chloride, including magnesium chloride, magnesium sulfate, and ammonium sulfate. Sodium chloride concentration may be from 100 mM to 1000 mM. If salt is not included, nonspecific staining of nonphosphoproteins is increased. Nonspecific staining is reduced to low levels by extensive washing with 50 mM sodium acetate (pH 4.0). Other buffers besides sodium acetate may be used, including formate and 2-(N-morpholino)ethanesulfonic acid. If 1, 2-propanediol is omitted, the background staining of the gel is increased but phosphoproteins are still selectively stained. Effective pH ranges of the acidic buffers are in the range of 3.0 to 5.0.

Example 3: Serial Dichromatic Detection of Phosphoproteins and Total Protein in SDS Polyacrylamide Gels.

After detection of the phosphoproteins as in Example 2, the gel was incubated overnight with 60 ml SYPRO™ Ruby protein gel stain with gentle orbital shaking, typically 50 rpm. The gel was then incubated in 7% acetic acid, 10% methanol for 30 minutes, also at 50 rpm. The orange signal from the phosphorylated and non-phosphorylated proteins was collected with a standard CCD camera-based imaging system with 300 nm UV light excitation and a 600 nm bandpass filter.

Example 4: Detection of phosphopeptides in a polyacrylamide gel.

Peptides generated by a trypsin digestion of bovine milk β-casein were separated by electrophoresis in a Novex® Tricine gel (16% polyacrylamide, Invitrogen™ life technologies). After electrophoresis the gel was fixed for 1 hour in 100 ml 40 % methanol, 10 % acetic acid, and then fixed for 1 hour in 100 ml of 40 % methanol, 0.82 M sodium acetate, 0.5% glutaraldehyde. The gel was washed with three changes of water, and then incubated for 100 minutes in 30 ml staining solution containing 50 mM sodium acetate, pH 4.0, 500 mM sodium chloride, 1 μM compound 2, 1 μM gallium chloride. The gel was then washed with three changes of 50 mM sodium acetate in 75 minutes. Images were acquired on a Fujifilm FLA 3000 laser scanner with 532 nm excitation and 580 nm bandpass emission filter and data displayed using the Image Gauge Analysis software. The two known phosphopeptides that result from a trypsin digest of β-casein were visible as prominent bands on the gel. The gel was then stained with 60 ml SYPRO™ Ruby protein gel stain (Molecular Probes, Eugene, Oregon) by incubating the gel overnight in the stain, and then incubating the gel in 7% acetic acid, 10% methanol for 30 minutes.

Example 5: Detection of Phosphoproteins in Isoelectric Focusing Gels

Isoelectric focusing (IEF) can be performed utilizing a variety of pre-cast and laboratory prepared gels that employ different chemistries to generate a pH gradient. In this instance, Ampholine PAG plates were run horizontally for 1500 volt-hours using a Multiphor II electrophoresis unit (Amersham-Pharmacia Biotech, Uppsala, Sweden) per the manufacturer's instructions. The gels were fixed in 100 ml 45% methanol, 5% acetic acid overnight. The gels were then washed with several changes of equal volumes of water, and incubated for 130 minutes in 50 ml of staining solution containing 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 3.0), 1000 mM NaCl, 1 μM compound 2, and 1 μM gallium chloride. The gels were washed with 50 ml 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 3.0), 1000 mM NaCl twice for 30 minutes per wash, and then in 50 mM 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 3.0). Images were acquired as described in Example 2.

Example 6: Detection of Phosphoproteins in Two-Dimensional Gels

A human MRC-5 lung fibroblast cell lysate protein mixture (150 μg) was diluted into urea buffer (7 M urea, 2 M thiourea, 2% CHAPS, 1% Zwittergent 3-10, 0.8% carrier ampholytes

(3-10), 65 mM DTT) and applied on a first dimension IPG strip (3-10 non linear, 18 cm). After overnight rehydration the strips were covered with mineral oil and the proteins were focused for 75, 000 volts total. IPG strips were then laid on top of 1 mm thick, 20 cm X 20 cm, 12.5% T, 2.6% C polyacrylamide gels containing 375 mM Tris-base, pH 8.8 and SDS-polyacrylamide gel electrophoresis was performed according to standard procedures except that the cathode electrode buffer was 50 mM Tris, 384 mM glycine, 4% SDS, pH 8.8 while the anode electrode buffer was 25 mM Tris, 192 mM glycine, 2% SDS, pH 8.8. After the second dimension electrophoresis, gels were fixed in 750 ml 45% methanol, 5% acetic acid for 20 hours. Gels were washed twice, 75 minutes per wash, with water and then put in 500 ml staining solution. The staining solution contained 50 mM sodium acetate, pH 4.0, 250 mM sodium chloride, 20% v/v 1, 2-propanediol, 1 μM compound 2, 1 μM gallium chloride. 500 μL of compound 2, in stock solution at 1mM and 500 μL of gallium chloride, in stock solution at 1 mM were added to 9 ml water. This mixture was then added to 490 ml of the staining buffer. The gel was incubated for 8 hours in the staining mixture; the stain was decanted and the gels were washed with 3 changes of 800 ml 50 mM sodium acetate, pH 4.0, 30 to 40 minutes per wash, and then washed overnight in 1 liter 50 mM sodium acetate, pH 4.0. Images were acquired on a Fujifilm FLA 3000 laser scanner with 532 nm excitation and 580 nm bandpass emission filter and data displayed using the Image Gauge Analysis software. Images of phosphoproteins were displayed as dark spots. Proteins not containing phosphate were not stained or were very lightly stained relative to the phosphoproteins. When gels were stained as above but with $GaCl_3$ omitted from the staining solution phosphoproteins were not selectively stained, and could not be distinguished from background or light nonspecific staining.

Example 7: Serial Dichromatic Detection of Phosphoproteins and Total Protein in Two-Dimensional Gels.

Electrophoresis and phosphoprotein detection was performed as in Example 4. After detection of the phosphoproteins, the gel was stained with 500 ml SYPRO™ Ruby protein gel stain by incubating the gel overnight in the stain, and then washing the gel in 7% acetic acid, 10% methanol for two changes, at 30 minutes each wash. Images were acquired as described in Example 2. Alternatively, the orange signal from the phosphorylated and

nonphosphorylated proteins is collected with a standard CCD camera-based imaging system with 300 nm UV light excitation and a 600 nm bandpass filter.

Example 8: Detection of Phosphoproteins Electroblotted to PVDF or Nitrocellulose Membranes

Proteins of interest were separated by SDS-polyacrylamide electrophoresis and transferred to PVDF membrane using standard procedures, and the membrane was allowed to air dry. The PVDF membrane was quickly dipped in 100% methanol, washed with a solution of 40% methanol, 5% acetic acid for 15 minutes, and with two changes of water for 10 minutes each. The blot was then added to a staining solution and incubated for 80 minutes at room temperature with gentle orbital shaking. The staining solution contained 50 mM sodium acetate, pH 4.0, 500 mM sodium chloride, 1 μM compound 1 or compound 4, and 1 μM gallium chloride. Typically, 60 μl of the labeled BAPTA, in stock solution at 1mM and 60 μl of gallium chloride, in stock solution at 1 mM were added to 540 μL water. This mixture was then added to 29.5 ml of the staining buffer. Alternatively the labeled BAPTA and the gallium chloride may be added separately, directly to 30 ml of the staining diluent. After incubation in staining solution, the gel was washed with 50 ml 50 mM sodium acetate; pH 4.0, 2 washes of 30 to 50 minutes each.

Images were acquired with a standard CCD camera imaging system (BioRad FluorS Max) with a reflective 300 nm UV light source, and a 465 nm bandpass emission filter for compound 4. Proteins not containing phosphate were not stained or were very lightly stained relative to the phosphoproteins. When the blot was stained as above but with $GaCl_3$ omitted from the staining solution, phosphoproteins were not selectively stained, and could not be distinguished from background or light nonspecific staining. For imaging with compound 1, the wet blots were placed face down in the Fujifilm FLA 3000 laser scanner with 473 nm excitation laser and 520 nm bandpass emission filter, and data displayed using the Image Gauge Analysis software.

Example 9: Dichromatic detection of phosphoproteins and total protein electroblotted to PVDF membrane.

Serial dichromatic detection of phosphoprotein and total protein on PVDF membrane was accomplished by post-staining the blot stained and imaged to detect phosphoprotein as in

Example 8 (above) with SYPRO™ Ruby protein blot stain (Molecular Probes, Eugene, Oregon) to detect total protein. The blot was floated face down on a solution of 10% methanol, 7% acetic acid for 15 minutes followed by face staining with SYPRO™ Ruby dye for 15 minutes. The blot was washed face down on water, 3 changes in 10 minutes. The membrane was allowed to air dry. The fluorescent signal from total proteins was acquired with a standard CCD camera imaging system (BioRad FluorS Max) with a reflective 300 nm UV light source and a 610 nm longpass filter.

Dichromatic staining was achieved by image acquisition with a standard CCD camera imaging system (BioRad FluorS Max) with a reflective 300 nm UV light source and a 465 nm bandpass emission filter as in Example 8. The signal from phosphoprotein stained with compound 4/Ga (III) could be distinguished from the signal from total protein stained with SYPRO™ Ruby, not detected with the 465 nm bandpass filter.

For compound 1, SYPRO™ Ruby staining and image acquisition as above reveals fluorescent signal from total protein, revealing the phosphoproteins as a subset when the SYPRO™ Ruby image is compared to the fluorescent image obtained as in Example 6, above.

Example 10: Detection of Phosphatase Activity

Phosphoproteins and non-phosphorylated proteins were incubated with commercially available calf intestinal alkaline phosphatase at 37° C for 30 minutes under standard conditions. Control digests were done under the same conditions with no enzyme. Suitable test proteins include bovine α-casein, ovalbumin, and pepsin as phosphoproteins; and bovine serum albumin, chicken egg white lysozyme, and soybean trypsin inhibitor as non-phosphorylated proteins. Electrophoresis was performed as per Example 2, with control (undigested) and phosphatase-treated samples loaded pairwise, 1250 ng protein per lane. Phosphoprotein detection was performed as per Example 2 above, with images taken 90 minutes after staining and again after overnight washing. An additional gel was stained as per Example 2 but with no gallium chloride in the stain. For the gel stained with the full staining mixture, comparisons of the control, undigested sample proteins showed that the phosphoproteins appeared as dark bands according to the software display and the nonphosphoproteins were not stained or were only very lightly stained. For the gel stained

with the formulation lacking gallium chloride, phosphoproteins showed the same degree of no staining or only very light staining as the nonphosphoproteins, and this level of signal was the same as the nonphosphoproteins in the gels stained with the full formulation including gallium chloride. Comparison of the pairwise phosphoproteins in the fully stained gel showed that the signal from the alkaline phosphatase treated sample was significantly less than the signal from the undigested control. The very light signal from the nonphosphoproteins, if detectable, was the same for the control and enzyme-treated samples.

After detection of the phosphoproteins, the gel was stained for total protein with SYPRO™ Ruby protein gel stain as per Example 2 and images of SYPRO™ Ruby staining acquired as per Examples 3 and 7. The signal for total protein staining was similar for the pairwise control and digested samples for both gels, indicating that the reduced signal from alkaline phosphatase treated phosphoprotein samples was not due to protein degradation.

Example 11: Detecting Kinase Activity

Bovine muscle myosin light chain was incubated with commercially available cloned calmodulin-dependent protein kinase II (New England BioLabs) according to the manufacturer's instructions, with 100 mM adenosine triphosphate (ATP) and the supplied buffer components. A parallel, control incubation was done with no enzyme. A sample of each reaction mixture was loaded in adjacent lanes and analyzed by electrophoresis as in Example 2. The gels were fixed in 100 ml 45% methanol, 5% acetic acid for 60 minutes. The gels were then washed with several changes of water. One gel was incubated for 110 minutes in 30 ml of staining solution containing 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 3.0, 1000 mM NaCl, 1 μM compound 2, 1 μM gallium chloride. The other gel was incubated in an identical solution, minus gallium chloride. The gels were washed with 50 ml 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 3.0, 1000 mM NaCl twice for 30 minutes per wash, and then in 50 mM 50 mM 2-(N-morpholino)ethanesulfonic acid, pH 3.0. Image acquisition for phosphoprotein detection was done as in Example 2 and serial dichromatic detection of phosphoproteins and total protein was done as in Example 3.

Staining for total protein revealed identical profiles of 3 major bands in both lanes. Staining for phosphoprotein revealed one major band in both lanes, with the signal from the band in

the lane corresponding to the reaction containing the enzyme 3.4-fold greater than the no-enzyme control.

Example 12: TRAIL/Apo2L detection

To determine the cell signaling factors involved in TRAIL/Apo2L mediated apoptosis, a proteomics approach involving 2-D gel electrophoresis and mass spectrometry is used. This approach involves comparing 2-D gels of colon cancer cells (Colo205) treated and not treated with a soluble fragment of TRAIL/Apo2L (amino acids 114-281) for various lengths of time ranging from several seconds to several hours. To assist in comparison of 2-D gels, compound 7 bound to gallium ions is used, in conjunction with the total protein stain SYPRO™ Ruby. Since cell signaling often involves protein phosphorylation, the use of compound 7 highlights spots likely to be involved in death receptor signaling or apoptotic signaling. Protein spots that are significantly different between the TRAIL/Apo2L treated and untreated Colo205 cells are identified by subsequent mass spectrometry analysis.

Example 13: Phosphopeptide Precipitation Using Fluorescent Labeled BAPTA

A mixture of two non-phosphorylated peptides (Angiotensin I and II) and two phosphorylated peptides (pT/pY and RII) were combined (5 ug each) in a final volume of 100 ul containing 100 mM sodium acetate, pH 4.0, 0.2 mM $GaCl_3$ and 0.1 mM compound 9. The mixtures were vigorously vortexed for 1 hour at room temperature and then centrifuged in a microfuge at full speed for 5 minutes. The supernatants were removed and stored. The pellets were resuspended by triturating with a micropipet tip in 100 ul wash buffer (100 mM sodium acetate, pH 4.0, 0.2 mM $GaCl_3$). The samples were again centrifuged for 5 minutes and the supernatant wash components were saved for analysis. The pellets were dissolved in (10-50% acetonitrile, 0.1% TFA for further analysis by HPLC or MALDI mass spectrometry. Pellets can also be dissolved in various different basic solutions of choice.

If labeled BAPTA removal is required after precipitation, any biotinylated fluorescent labeled BAPTA, such as compound 10, can be used in the precipitation procedure. After separation of the pellet phosphopeptides from the BAPTA complex using organic or base treatment, the labeled BAPTA can be removed using an immobilized streptavidin support (e.g. streptavidin-agarose or streptavidin magnetic beads.)

Example 14: Detection of a phosphoprotein in solution by fluorescence polarization.

A solution containing 1.0 μM compound 2 was incubated in 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 3.0 to 3.5), 500 mM NaCl at room temperature in parallel to solutions containing, in addition, (a) 1 μM gallium chloride, (b) 20 μM ovalbumin, or (c) 1 μM gallium chloride and 20 μM ovalbumin. The fluorescence intensity of the resulting solutions was then measured using a rotating emission polarizer in a fluorescence spectrophotometer with excitation at 530 nm and emission at 545 to 700 nm. The integrated emission spectra yielded anisotropy "r values" of: r = 0.05 +/- 0.02 (compound 2 plus gallium); r = 0.10 +/- 0.02 (compound 2 plus ovalbumin); r = 0.17 +/- 0.01 (compound 2 plus gallium plus ovalbumin), indicating gallium-dependent binding of the compound 2 to this phosphoprotein.

Example 15: Isolation and characterization of phosphopeptides from complex protein digests.

An unlabeled BAPTA-agarose column (compound 13 or 14) (typically 200 μl of medium) was charged with 0.1M $GaCl_3$ and washed with de-ionized H_2O until the pH of the flow-through material approached 7.0. The column was then equilibrated with 5 column volumes of binding buffer (100 mM sodium acetate buffer (pH 3.0)). The phosphopeptide mixture was vacuum dried in the SpeedVac (Savant) or similar instrument and dissolved in binding buffer. If the final pH of the peptide mixture is not 3.0, then it can be adjusted with 1-10 M acetic acid as appropriate. The protein digest (1-5 mg/ml) was applied in 1 column volume or less (but no less than half the column volume) and followed with 2 column volumes of binding buffer. Flow-through (FT) fractions were combined and stored for further analysis. The column was washed with 2 column volumes of 100 mM sodium acetate (pH 7.0), 500 mM NaCl, 10% acetonitrile followed by 1 column volume of sodium acetate (pH 7.0). The FT fractions were combined and stored for further analysis. Bound peptides were eluted with 3 separate column volumes of saturated $Ba(OH)_2$ that are collected in a single tube. The pH of the resulting elution fraction was greater than pH 11.0, and when it was not, it was immediately adjusted with saturated barium hydroxide ($Ba(OH)_2$). The elution fraction was incubated for 90 minutes at 30°C. After incubation, the sample was divided into 2 portions, one of which was neutralized to pH 5.0-7.0 with glacial acetic acid and stored frozen. One half volume of de-ionized water is added to the other tube followed by the addition of a concentrated nucleophilic thiol or amine (methylamine, cystamine or β-mercaptoethylamine) to achieve a final concentration of 0.1 –0.5 M in a volume not exceeding 1/6 of the starting

sample/H_2O volume. The reaction mix was incubated for an additional 60 minutes at 30°C, then neutralized to pH 5.0-7.0 with glacial acetic acid. For MALDI-TOF mass spectrometry analysis, peptides were purified from samples using C18 ZipTips (Millipore) using standard protocols, vacuum dried in a SpeedVac dryer and dissolved in 50% acetonitrile and 0.1% TFA. An equal volume of 10 mg/ml MALDI matrix (α-cyano-5-hydroxycinnamic acid) in the same solvent was added. The solution was mixed thoroughly and 1 µl is spotted onto the MALDI target.

Differential mass weight analyses of both peptide fractions resulted in the determination of the number of phosphorylation sites on the peptides, as well as the nature of the phosphoamino acids. Under the conditions used, only phosphoserine residues undergo elimination and nucleophilic addition (loss of phosphoric acid -98 amu, + mass weight of nucleophilic addition reagent). Phosphothreonine residues undergo elimination only (loss of phosphoric acid only, -98 amu) and phosphotyrosine residues remain unchanged, as phosphotyrosine is stable in strong base.

Example 16: Quantitating the number of phosphates on ovalbumin.
Solutions of 1 µM and 4 µM ovalbumin were incubated in 75 mM Sodium Acetate (pH 4.0), 140 mM NaCl, 0.1 µM compound 4, and 1.7 µM gallium chloride at room temperature for 5-10 minutes. The fluorescence intensity of the resulting solution was then measured at 410 nm in a fluorescence spectrophotometer and compared to a standard phosphate calibration curve to determine the number of phosphates on ovalbumin. The standard phosphate calibration curve was produced by equilibrating known concentrations (1, 2, 4, 6, 8, and 10 µM) of a 19 amino acid phosphoserine-containing peptide in 75 mM Sodium Acetate (pH 4.0), 140 mM NaCl, 0.1 µM compound 4, and 1.7 µM gallium chloride and measuring the fluorescence intensity at 410 nm. Next the fluorescence intensity was graphed versus the known concentration of phosphopeptide. The fluorescence intensity from the solution containing ovalbumin was then compared to the standard curve to reveal 2 µM and 8 µM phosphate. Finally, when accounting for the protein's concentration resulted in the determination of two phosphate groups per molecule of ovalbumin.

Example 17: Phospholipid detection using labeled BAPTA compound

To test the detection of phospholipids with the present invention, different phospholipids were spotted onto a nitrocellulose membrane. The phospholipids were obtained from Echelon Research Labs in a format called a PIP Array™, which contains 8 different phosphoinositides (PtdIns) at 7 different concentrations. PIP Arrays™ were used for determining the sensitivity limits of the invention for detecting phospholipids.

PIP Array™

1. PtdIns	100	50	25	12.5	6.3	3.2	1.6 pmol
2. PtdIns (3) P							
3. PtdIns (4) P							
4. PtdIns (5) P							
5. PtdIns (3,5) P2							
6. PtdIns (4,5) P2							
7. PtdIns (3,4) P2							
8. PtdIns (3,4,5,) P3							

One PIP Array™ was washed in 50 mM sodium acetate (pH 4.0) for 15 min. After the wash, the PIP Array™ was incubated in 50 mM sodium acetate (pH 4.0), 20 % 1,2 propanediol, 500 mM NaCl, 1 μM compound 1, and 1 μM $GaCl_3$ for 1 hour, by incubating the array at 100-150 RPM on an orbital shaker. After incubating the PIP Array,™ the array was washed 3 times in 50 mM sodium acetate (pH 4.0) for 15 minutes each at 100-150 RPM on an orbital shaker to remove unbound dye and reduce the background fluorescence. An image of the PIP Array™ was generated using a Laser scanner (Fuji FLA 3000) with an excitation wavelength of 473 nm and an emission filter of 520 nm. Of the 8 phosphoinositides 4 gave a strong positive signal. These included phosphatidic acid, phosphoinositide (4,5) P_2, phosphoinositide (3,4) P_2 and phosphoinositide (3,4,5) P_3. The strongest signal was obtained with phosphoinositide (3,4) P_2 followed by phosphoinositide (4,5) P_2 and then phosphoinositide (3,4,5) P_3.

Example 18: Synthesis of quinazolinone labeled BAPTA (Q-BAPTA) compounds (Compounds 7 and 15)

Preparation of 5-Fluoro-Q-BAPTA (Compound 7): a catalytic quantity of p-toluenesulfonic acid was added to a colorless solution of anthranilamide (29 mg, 0.21 mmol) and 5'-fluoro-5-formyl-4-hydroxy-BAPTA tetramethylester (128 mg, 0.21 mmol) in 10 mL dichloroethane/5 mL ethanol. The resulting solution was stirred at reflux overnight, then cooled to room temperature. Next, p-chloraniline (57 mg, 0.23 mmol) was added. After 2 hours, the volatiles were removed *in vacuo* and the residue purified by flash chromatography using 5% methanol/chloroform as an eluant to provide the tetramethylester of compound 7 as 50 mg of a light amber immobile oil; m/z 711 (710 calc for $C_{34}H_{34}N_4O_{12}F$).

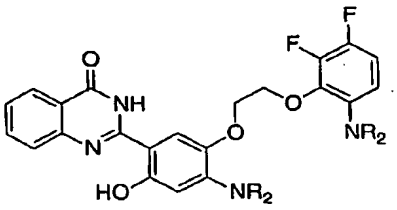
To a green solution of the tetramethylester of compound 7 (50 mg, 0.07 mmol) in 1:1 dioxane:methanol (5 mL), a 1M aqueous solution of KOH (0.56 mL, 0.56 mmol) was added. The resulting yellow solution was stirred overnight, then concentrated *in vacuo*. The residue was purified by chromatography on Sephadex LH-20 using water as eluant, generating 53 mg of compound 7 as a yellow powder; m/z (positive mode) 655 (651 calculated for $C_{30}H_{23}N_4O_{12}F$).

Preparation of 5,6-Difluoro-Q-BAPTA.(Compound 15) 5,6-Difluoro-4'-hydroxy-5'-formyl BAPTA tetramethylester (0.100 g, 0.163 mmol) and anthranilamide (0.022 g, 0.162 mmol) were dissolved in a mixture of methylene chloride (10 mL) and ethanol (5 mL). P-TsOH (5 mg) was added to the solution and the reaction mixture was stirred under reflux for 3 hrs. Chloraniline (0.044 g, 0.18 mmol) was added to the resulting solution. The mixture was refluxed for 2 more hours and evaporated. The crude product was purified using preparative TLC, using 2:1 chloroform-ethyl acetate as eluant. The main component (R_f = 0.5) was isolated and washed out of silica gel with ethyl acetate. The resulting solution was concentrated to give 5,6-difluoro-Q-BAPTA tetramethylester as a white powder (0.029 g, 24%).

5,6-Difluoro-Q-BAPTA tetramethylester (0.027 g, 0.037 mmol) was dissolved in a mixture of 1 mL of methanol and 1 mL of dioxane. 1M KOH (1 mL) was added to the resulting solution and the reaction mixture was kept overnight at RT. Volatiles were evaporated, and the crude product was re-dissolved in water and put onto a Sephadex LH-20 column. The

column was eluted with water. Fractions containing pure product were combined and lyophilized to give 5,6-difluoro-Q-BAPTA potassium salt (Compound 15) as a yellow powder (0.021 g, 69%).

Compound 15



Example 19: Synthesis of (BODIPY-BAPTA) compounds (Compound 8 and 16)

Preparation of BODIPY FL BAPTA-5F (Compound 8): To a solution of 5-fluoro-BAPTA tetramethylester (1.00 g, 1.82 mmol) in 9 mL acetic anhydride, with ice-bath chilling, was add 70% nitric acid (0.15 mL, 2.3 mmol). After 10 minutes the reaction solution was poured into 30 mL aqueous NaOAc, then saturated sodium bicarbonate solution was carefully added. The resulting mixture was extracted with chloroform (2x30 mL). The extract was washed with brine, dried over sodium sulfate, and concentrated to an amber residue which was purified by flash chromatography using ethyl acetate/hexanes as the mobile phase to afford 5-nitro-5'-fluoro-BAPTA, tetramethylester as 0.43 g yellow powder.

To a solution of 5-nitro-5'-fluoro-BAPTA, tetramethylester (0.43 g, 0.72 mmol) in 1:1 methanol/dioxane (10 mL) was added 1M KOH (5.8 mL, 5.8 mmol). The resulting solution was stirred overnight, then concentrated in vacuo. The residue was dissolved in 10 mL water, and the pH lowered to 2 with aqueous HCl. The resulting precipitate was collected and dried in vacuo to give 5-nitro-5'-fluoro-BAPTA free acid as 0.31 g of yellow powder.

A solution of 5-nitro-5'-fluoro-BAPTA free acid (0.31 g, 0.58 mmol) in 30 mL methanol was shaken over 10% Pd/carbon (0.15 g) under 38 psi hydrogen gas for 6 hours, then filtered and concentrated to give 5-amino-5'-fluoro-BAPTA free acid as 0.26 g of a colorless powder.

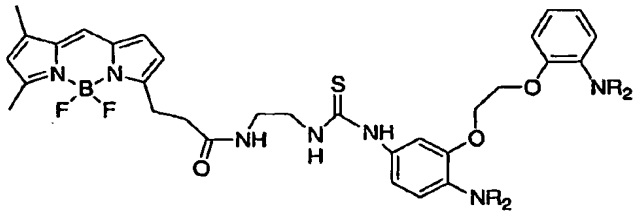
A solution of BODIPY FL free acid (Molecular Probes, Inc. 2183, 27 mg, 0.09 mmol) in 5 mL anhydrous THF was treated with an oxalyl chloride (0.20 mmol) and

diisopropylethylamine (DIEA, 0.20 mmol) under argon. After stirring for 15 minutes, the volatiles were removed in vacuo. The residue was dissolved in 3 mL anhydrous dioxane, and the resulting solution was slowly added to a solution of 5-amino-5'-fluoro-BAPTA free acid (50 mg, 0.10 mmol) in 5 mL water which had been pH-adjusted to pH=9.5 with aqueous sodium carbonate. The resulting solution was stirred overnight, then the organic volatiles removed by rotary evaporation. The resulting solution was purified by column chromatography on Sephadex LH-20 using water as eluant to afford BODIPY FL BAPTA-5F (Compound 8), sodium salt as 41 mg of an orange powder after lyophilization of the combined pure product fractions.

Preparation of BODIPY FL-EDA-BAPTA (Compound 16)

The pH (6.0) of a solution of BODIPY FL ethylenediamine hydrochloride salt (15 mg, 0.04 mmol, Molecular Probes) in 3 mL water was raised to 7.6 by drop-wise addition of aqueous sodium bicarbonate. A solution of 5-isothiocyanato-BAPTA free acid (22 mg, 0.04 mmol) in 2 mL dioxane was added. The resulting pH (3.0) was raised to 9.5 with aqueous sodium bicarbonate, and the resulting orange solution stirred at room temperature overnight. The reaction solution was concentrated via rotary evaporation to a volume of 2 mL, and the resulting solution loaded onto a column of Sephadex LH-20. Elution with water gave BODIPY FL-EDA-BAPTA sodium salt (compound 16) as 17 mg of a fine orange powder after lyophilization of combined pure product fractions.

Compound 16

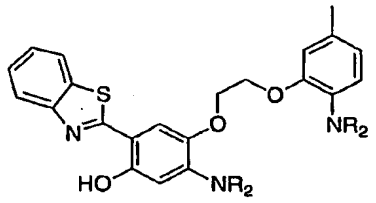


Example 20: Synthesis of 4-Hydroxy-5-benzothiazolyl-BAPTA (Compound 17)

A solution of 4-hydroxy-5-formyl-5'-methyl BAPTA, tetramethylester (0.40 g, 0.68 mmol) and 2-aminothiophenol (75 mg, 0.70 mmol) in DMSO (5 mL) was heated at reflux for 15 minutes. After cooling the yellow solution was diluted with 50 mL water. The resulting yellow precipitate was collected by filtration and dried, then purified by flash chromatography using ethyl acetate/hexanes as eluant to give 4-hydroxy-5-benzothiazolyl-BAPTA tetramethylester as 0.22 g yellow foam.

To a solution of 4-hydroxy-5-benzothiazolyl-BAPTA tetramethylester (0.21 g, 0.30 mmol) in 1:1 methanol/dioxane (10 mL) was added 1M KOH (3.0 mL, 3.0 mmol). The resulting solution was stirred for 3 hours, then concentrated in vacuo. The residue was purified by column chromatography on Sephadex LH-20 using water as eluant to give compound 17 as 0.13 g of a yellow-green powder after lyophilization of pooled pure product fractions.

Compound 17



Example 21: Synthesis of BAPTA-agarose compounds (Compounds 13 and 14)

Preparation of BAPTA-agarose (compound 13)

A solution of 5-isothiocyanato-BAPTA free acid (65 mg, 0.12 mmol, US Patent No. 5,453,517) in 3 mL anhydrous DMF was added to a slurry of aminoagarose (50% aqueous slurry, 16 µmol amine/mL, 6 mL, 96 µmole amine, Pierce) that had been diluted with 15 mL DMF. The reaction pH (3.5) was raised to 10 by the addition of DIEA (1.5 mL). The resulting light brown mixture was stirred at room temperature for 48 hours, then centrifuged. The resulting BAPTA-agarose (compound 13) pellet was rinsed with acetone (2x) and water (2x), then suspended in water.

Preparation of BAPTA-5F-agarose (compound 14)

A solution of 5-amino-5'-fluoro-BAPTA free acid (0.26 g, 0.51 mmol) in 12 mL aqueous HCl was diluted with 12 mL chloroform, then treated with thiophosgene (3 mL). The resulting orange mixture was stirred vigorously at room temperature overnight, then the organic volatiles removed by rotary evaporation. The resulting mixture was centrifuged, giving as the pellet a brown gum which was dried in vacuo then dissolved in 2 mL anhydrous THF. Addition of 20 mL ethyl acetate caused a precipitate to form, which was isolated by centrifugation to give 5-fluoro-5'-isothiocyanoto-BAPTA free acid as a light grey-brown powder.

A solution of 5-fluoro-5'-isothiocyanato-BAPTA free acid (25 mg, 0.05 mmol) in 1 mL anhydrous DMF was added to a slurry of aminoagarose (50% aqueous slurry, 16 µmol amine/mL, 2 mL, 32 µmole amine, Pierce) that had been diluted with 5 mL DMF. The reaction pH (3) was raised to 10 by the addition of a few drops of DIEA. The resulting light brown mixture was stirred at room temperature for 48 hours, then centrifuged. The resulting BAPTA-5F-agarose (compound 14) pellet was rinsed with acetone (2x) and water (2x), then suspended in water.

Example 22: Synthesis of biotinylated BAPTA compounds (Compounds 9, 12 and 18)

Preparation of Biotin-BAPTA-5F (compound 12)

A solution of 5-nitro-5'-fluoro-BAPTA, tetramethylester was reduced by catalytic hydrogenation over 10% Pd/C in ethyl acetate. To a solution of the resulting 5-amino-5'-fluoro-BAPTA, tetramethylester (0.10 g, 0.18 mmol) in anhydrous dichloromethane/THF (4:1, 5 mL) was added glutaric anhydride (40 mg, 0.36 mmol) and catalytic DMAP. The resulting solution was stirred overnight, then concentrated in vacuo. The residue was purified by flash chromatography using 10% methanol/chloroform to give the glutaramide of 5-amino-5'-fluoro-BAPTA, tetramethylester as 0.13 g of a light brown clear immobile oil.

To a solution of the glutaramide of 5-amino-5'-fluoro-BAPTA, tetramethylester (0.18 mmol) in 5 mL anhydrous THF and 5 mL anhydrous acetonitrile was added N-hydroxysuccinimidyluronium tetrafluoroborate (108 mg, 0.36 mmol). After two hours a solution of biotin ethylendiamine hydrobromide (66 mg, 0.18 mmol, Molecular Probes) and

DIEA (0.05 mL) in 2 mL anhydrous DMF was added. After stirring overnight, the volatiles were removed in vacuo. The residue was triturated with water (15 mL), and the resulting precipitate was collected, rinsed with water, and dried in vacuo to give Biotin-BAPTA-5F tetramethylester as 0.10 g of a grey powder.

To a grey solution of Biotin-BAPTA-5F tetramethylester (0.10 g, 0.11 mmol) in 1:1 methanol/dioxane (4 mL) was added 1M KOH (1.0 mL, 1.0 mmol). The resulting light brown solution was stirred overnight, then the volatiles removed in vacuo. The residue was purified by column chromatography over Sephadex LH-20 using water as eluant, which gave Biotin-BAPTA-5F (compound 12) potassium salt as a colorless powder after lyophilization of combined pure product fractions.

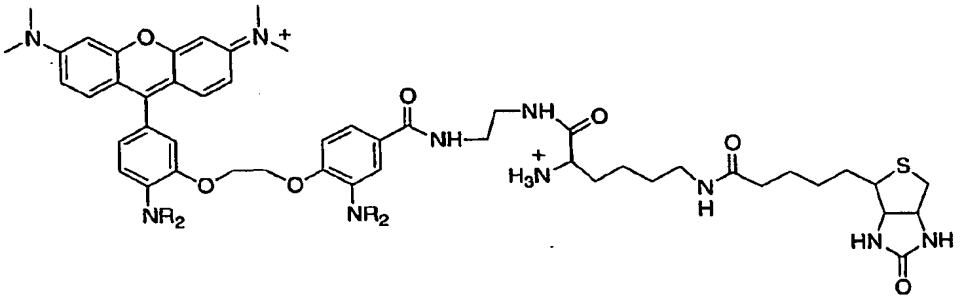
Preparation of Rhod-biocytin (Compound 18)

To a 0.5M solution of 4-(succinimidyloxycarbonyl)-rhod tetramethyl ester in anhydrous THF is added 1.1 eq. of N-t-BOC-ethylenediamine and 1.1 eq. of DIEA. The resulting solution is stirred for 30 minutes, then concentrated in vacuo. The residue is purified by flash chromatography using chloroform/methanol/acetic acid as eluant. The purified carbonate is dissolved in dichloromethane (0.5M) and treated with trifluoroacetic acid (20 eq.). The resulting solution is stirred 30 minutes, then concentrated in vacuo to give the ethylenediamine carboxamide of 4-carboxy-rhod tetramethyl ester.

To a 0.5M solution of the ethylenediamine carboxamide of 4-carboxy-rhod tetramethyl ester in DMF is added N-t-BOC-biocytin succinimidyl ester (1.5 eq., described in Wilbur et al., *Bioconjugate Chemistry* **2000**, 11: 584-98) and DIEA (1.5 eq.). The resulting solution is stirred at room temperature until TLC indicates consumption of the fluorescent starting material. The volatiles are removed in vacuo, and the residue is purified by flash chromatography using chloroform/methanol/acetic acid as eluant to give N-t-BOC-rhod-biocytin tetramethyl ester.

A 0.5M solution of N-t-BOC-rhod-biocytin tetramethyl ester in 1:1 methanol/dioxane is treated with 12 eq. of 1M KOH. The resulting solution is stirred overnight at room temperature, then concentrated in vacuo. The residue is purified by column chromatography on Sephadex LH-20 using water as eluant to give compound 18 as a red powder after lyophilization of combined pure product fractions.

Compound 18



Preparation of Rhod-4-biotin-BAPTA (Compound 9)

A suspension of (2'-nitrophenoxy)-2-chloroethane (20.15 g, 0.10 mol), methyl (4-hydroxy-3-nitro) benzoate (21.67 g, 0.11 mol), and K_2CO_3 (27.60 g, 0.20 mol) was stirred at 130 °C for 16 h, cooled to room temperature, and poured into ice water (1.2 L). The precipitate was filtered off, washed with H_2O and dried to give (4'-methoxycarbonyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane, 32.00 g (88%) as a yellow solid. 4'-methoxycarbonyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane (20.0 g, 55.2 mmol) was hydrogenated over 10% Pd/C (3.0 g) in DMF (300 mL) at 40 psi for 5 h. The mixture was filtered from catalyst through Celite on a fritted glass filter. The filtrate was evaporated and ether (100 mL) was added. The product was filtered off, washed with ether (2 x 25 mL) to give compound 1'-amino-4'-methoxycarbonylphenoxy)-2-(2''-aminophenoxy)ethane, 13.2 g (79%) as off-white solid.

A mixture of 2'-amino-4'-methoxycarbonylphenoxy)-2-(2''-aminophenoxy)ethane (13.20 g, 44 mmol), methanol (50 mL), dioxane (50 mL), and 1N KOH (100 mL, 100 mmol) was stirred at 65 °C for 5 h, then overnight at room temperature. The mixture was evaporated and the residue suspended in H_2O (500 mL). Aqueous 1N HCl was added to achieve pH 5.0. The precipitated product was filtered, washed with H_2O, and dried on a filter for 4 h, then washed with ether (3 x 25 mL) to give compound (2'-amino-4'-carboxy-1'-phenoxy)-2-(2''-aminophenoxy)ethane, 12.50 g as an off-white solid.

Diphenyldiazomethane was prepared by vigorously stirring benzophenone hydrazone (6.66 g, 34 mmol) and yellow HgO (17.60 g, 80 mmol) in hexanes (200 mL) for 3 h. The mixture was filtered from inorganics, and the filtrate evaporated and the residue dissolved in acetone (50 mL). This solution was added to a suspension of 2'-amino-4'-carboxy-1'-phenoxy)-2-(2''-aminophenoxy)ethane (5.76 g, 20 mmol) in acetone. The resulting mixture was stirred for 16 h at 35 °C, then the excess of diphenyldiazomethane was decomposed with AcOH (2 mL) over 2 h. The mixture was evaporated, and the crude product purified by flash chromatography on a SiO_2 column (12 x 50 cm bed) using $CHCl_3$ as eluent to give (2'-amino-4'-diphenylmethoxycarbonylphenoxy)-2-(2''-aminophenoxy)ethane, 6.80 g (75%) as an off-white solid.

A mixture of 2'-amino-4'-diphenylmethoxycarbonylphenoxy)-2-(2''-aminophenoxy)ethane (8.28 g, 18.24 mmol), DIEA (16.3 mL, 94 mmol), methyl bromoacetate (35.3 mL, 376 mmol), and NaI (1.50 g, 10 mmol) in MeCN (400 mL) was refluxed under stirring for 70 h, cooled to room temperature and evaporated. The residue was dissolved in $CHCl_3$ (500 mL), washed with 1% AcOH (3 x 200 mL), H_2O (200 mL), sat. NaCl (200 mL), filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (12 x 55 cm bed) using a gradient of 30-40% EtOAc in hexanes as eluent to give 4-diphenylmethoxycarbonyl-BAPTA tetramethyl ester, 10.01 g (74%) as a white solid.

To a solution of Vilsmeier reagent made from $POCl_3$ (5 mL, 50 mmol) in DMF (35 mL) was added a solution of 4-diphenylmethoxycarbonyl-BAPTA tetramethyl ester (3.71 g, 5 mmol) in DMF (15 mL). The mixture was stirred at 40 °C for 24 h, then another portion of Vilsmeier reagent (25 mmol) was introduced and the mixture stirred at 40 °C for 70 h. The mixture was cooled to room temperature and quickly poured into an ice-sat. K_2CO_3 mixture (1200 mL). After 1 h coagulation the precipitated product was filtered, washed with H_2O and dried to give 4-diphenylmethoxycarbonyl-5'-formyl-BAPTA tetramethyl ester, 3.78 g (98%) as a white solid.

A mixture of 4-diphenylmethoxycarbonyl-5'-formyl-BAPTA tetramethyl ester (2.90 g, 3.8 mmol), m-dimethylaminophenol (1.21 g, 8.8 mmol), and TsOH (100 mg,) in prop ionic acid (40 mL) was stirred at 68 °C for 20 h, then cooled to room temperature and poured into 3N NaOAc (600 mL). After 1 h coagulation the precipitated product was filtered, washed with

water, and dried to give 4-diphenylmethoxycarbonyl-dihydrorhod tetramethyl ester, 3.70 g (97%) as a purple-red solid.

A mixture of 4-diphenylmethoxycarbonyl-dihydrorhod tetramethylester (2.050 g, 2.0 mmol) and powdered chloraniline (0.492 g, 2.0 mmol) in $CHCl_3$ and MeOH (40 mL of each) was stirred for 2 h, filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (4 x 50 cm bed) using a gradient 5-6.5% MeOH in $CHCl_3$/ 1% AcOH as eluent to give a crude product, which was re-dissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 4-diphenylmethoxycarbonyl-rhod tetramethyl ester, 0.533 g (25%) as a dark purple solid.

To a solution of 4-diphenylmethoxycarbonyl-rhod tetramethyl ester (51 mg, 0.05 mmol) in dioxane (2 mL) and MeOH (1 mL) was added 1N KOH to give pH 12.0. The mixture was stirred for 20 h, then the pH adjusted to 9.0 with 0.1 N HCl. The mixture was evaporated and the residue purified by column chromatography on Sephadex LH-20 (2.6 x 90 cm bed) using H_2O as eluant. Pure product fractions were combined and lyophilized to give 4-carboxy-rhod tetrapotassium salt, 26 mg (54%) as a red-purple solid.

To a solution of 4-diphenylmethoxycarbonyl-rhod tetramethyl ester (102 mg, 0.1 mmol) in $CHCl_3$ (10 mL) was added TFA (10 mL) and the resulting mixture stirred for 1 h, then evaporated and co-evaporated with $CHCl_3$ (3 x 10 mL). Ether (10 ml) was added to the residue and the precipitate was filtered and washed with ether (3 x 10 mL) to give 4-carboxy-rhod tetramethyl ester, 82 mg (96%) as a dark purple solid.

To a solution of 4-carboxy-rhod tetramethyl ester (80 mg, 0.093 mmol) in DMF (2 mL) was added DIEA (0.35 mL, 2 mmol) and dry trifluoroacetyl-N-hydroxysuccinimide (TFA-SE, 225 mg, 1 mmol). The mixture was stirred for 2 h, then more TFA-SE (113 mg, 0.5 mmol) was introduced and the mixture stirred for another 16 h. The mixture was diluted with $CHCl_3$ (50 mL), washed with 1% AcOH (3 x 20 mL), H_2O (25 mL), sat. NaCl (50 mL), filtered and evaporated. Ether (25 mL) was added to the residue, and the precipitated product filtered and washed with ether to give 4-succinimidyloxycarbonyl)-rhod tetramethyl ester, 86 mg (97%) as a dark purple solid.

To a solution of biotin-cadaverine (34 mg, 0.077 mmol, Molecular Probes, Inc.) in DMF (1 mL) and DIEA (0.055 mL, 0.40 mmol) was added a solution of 4-succinimidyloxycarbonyl)-rhod tetramethyl ester (36 mg, 0.038 mmol). The mixture was stirred for 3 h, diluted with $CHCl_3$ (200 mL), washed with 1% AcOH (3 x 150 mL), H_2O (100 mL), sat. NaCl (200 mL), filtered and evaporated. The residue was purified on two preparative TLC SiO_2 plates, using 12% MeOH and 2.5% AcOH in $CHCl_3$ as eluent to give 4-(N-(5''-biotinylaminopentane)aminocarbonyl)-rhod tetramethyl ester, 38 mg (79%).

To a solution of 4-(N-(5''-biotinylaminopentane)aminocarbonyl)-rhod tetramethyl ester (30 mg, 0.025 mmol) in MeOH (2 mL) and H_2O (1 mL) was added 1N KOH to give pH 12.0. The mixture was stirred for 20 h, then pH-adjusted to 8.5 with 0.1 N HCl. The mixture was evaporated and the residue purified on a Sephadex LH-20 column (1.6 x 40 cm bed) using H_2O as eluant and lyophilized to give Compound 9 (4-(N-(5''-biotinylaminopentane)aminocarbonyl)-rhod tetrapotassium salt) 30 mg (97%) as an orange-red solid.

Example 23: Synthesis of 4-(4'-(aminophenyl)-2-ethylamino)carbonylmethyl-rhod tripotassium salt (Compound 10).

A suspension of (2'-nitrophenoxy)-2-chloroethane (5.87 g, 29 mmol), methyl (4-hydroxy-3-nitro) phenyl acetate (6.15 g, 29 mmol), and K_2CO_3 (8.28 g, 60 mmol) was stirred at 120 °C for 16 h, cooled to room temperature, and poured into ice water (0.6 L). The precipitate was filtered off, washed with H_2O and dried to give (4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane, 4.49 g (41%) as a yellow solid.

4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane (9.6 g, 25.5 mmol) was hydrogenated over 10% Pd/C (1.0 g) in DMF (250 mL) at 40 psi for 16 h. The mixture was filtered from catalyst through Celite on a fritted glass filter. The filtrate was evaporated and the residue was purified by flash-chromatography on a SiO_2 column (8 x 25 cm bed) using a gradient of 25–35% EtOAc in hexanes to give (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane, 5.53 g (69%) of an off-white solid.

A mixture of 2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane (5.50 g, 17.4 mmol), methanol (40 mL), dioxane (40 mL), and 1M KOH (35 mL, 35 mmol) was stirred at 45 °C for 1 h, then overnight at room temperature. The mixture was evaporated and the residue was suspended in H_2O (100 mL). Aqueous 1N HCl was added to achieve pH 3.0. Precipitated product was filtered, washed with H_2O, and dried to give (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2''-aminophenoxy)ethane, 4.59 g (87%) of an off-white solid.

Diphenyldiazomethane was prepared by vigorously stirring benzophenone hydrazone (2.94 g, 15 mmol) and yellow HgO (8.80 g, 40 mmol) in hexanes (70 mL) for 5 h. The mixture was filtered from inorganics, and the filtrate was evaporated and the residue was re-dissolved in acetone (20 mL). This solution was added to the solution of the 2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2''-aminophenoxy)ethane (3.02 g, 10 mmol) in acetone (120 mL). The resulting mixture was stirred for 16 h at 35 °C, then the excess of diphenyldiazomethane was decomposed with AcOH (0.5 mL) over 2 h. The mixture was evaporated, and the crude product was purified by flash chromatography on a SiO_2 column (12 x 50 cm bed) using 1% MeOH in $CHCl_3$ as eluent to give (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane, 4.44 g (95%) as an off-white solid.

A mixture of 2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-aminophenoxy)ethane (2.12 g, 4.5 mmol), DIEA (4.0 mL, 23.5 mmol), methyl bromoacetate (8.8 mL, 94 mmol), and NaI (0.50 g, 4.7 mmol) in MeCN (90 mL) was refluxed under stirring for 70 h, cooled to room temperature and evaporated. The residue was dissolved in $CHCl_3$ (500 mL), washed with 1% AcOH (3 x 200 mL), H_2O (200 mL), sat. NaCl (200 mL), filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (4 x 40 cm bed) using a gradient of 30-40% EtOAc in hexanes as eluent to give 4-diphenylmethoxycarbonylmethyl-BAPTA tetramethyl ester, 2.82 g (83%) as a white solid.

To a solution of Vilsmeier reagent made from $POCl_3$ (1.5 mL, 30 mmol) in DMF (10 mL) was added a solution of 4-(diphenylmethoxycarbonylmethyl)-BAPTA tetramethyl ester (3.78 g, 5 mmol) in DMF (5 mL). The mixture was stirred for 24 h, then was quickly poured into an ice-sat. K_2CO_3 mixture (500 mL). The mixture was extracted with $CHCl_3$ (200 + 7x100 mL), dried over $MgSO_4$ and evaporated. The mixture of products was separated by column chromatography on SiO_2 (4 x 45 cm bed) using a gradient of 30-40% EtOAc in hexanes to

give aldehyde 4-(diphenylmethoxycarbonylmethyl)-5'-formyl-BAPTA tetramethyl ester, 1.65 g (42%) as a white solid.

A mixture of aldehyde 4-(diphenylmethoxycarbonylmethyl)-5'-formyl-BAPTA tetramethyl ester (784 mg, 1.0 mmol), m-dimethylamino phenol (301 mg, 2.2 mmol), and TsOH (20 mg, catalyst) in propionic acid (10 mL) was stirred at 65 °C for 20 h, then cooled down to room temperature and poured into 3N NaOAc (150 mL). After 1 h coagulation the precipitated product was filtered, washed with water, and dried to give 4-(diphenylmethoxycarbonylmethyl)-dihydro-rhod tetramethyl ester, 450 mg (46%) as a purple-red solid.

A mixture of 4-(diphenylmethoxycarbonylmethyl)-dihydro-rhod tetramethyl ester (420 mg, 0.43 mmol) and powdered chloraniline (122 mg, 0.5 mmol) in $CHCl_3$ and MeOH (20 mL of each) was stirred for 3 h, filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (4 x 50 cm bed) using a gradient of 5-7% MeOH in $CHCl_3$/ 0.5% AcOH as eluent to give a crude product, which was re-dissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 4-(diphenylmethoxycarbonylmethyl-5'-tetramethyl)-rhod tetramethyl ester, 275 mg (63%) as a dark purple solid.

To a solution of 4-(diphenylmethoxycarbonylmethyl-5'-tetramethyl)-rhod tetramethyl ester (250 mg, 0.25 mmol) in $CHCl_3$ (20 mL) was added TFA (20 mL) and the resulting mixture was stirred for 1 h, then evaporated and co-evaporated with $CHCl_3$ (3 x 30 mL). Ether (30 ml) was added to the residue and the precipitate was filtered, washed with ether (3 x 10 mL) to give 4-carboxymethyl-rhod tetramethyl ester, 200 mg (98%) as a dark purple solid.

To a solution of 4-carboxymethyl-rhod tetramethyl ester (128 mg, 0.15 mmol) in DMF (5 mL) and DIEA (0.40 mL, 2.2 mmol), dry TFA-SE (338 mg, 1.5 mmol) was added. The mixture was stirred for 16 h, then to the resulting solution a solution of 4'-aminophenylethylamine (0.4 mL, 4 mmol) and DIEA (0.4 mL, 2.2. mmol) was introduced. The mixture was stirred for 2h, diluted with $CHCl_3$ (500 mL), washed with 1% AcOH (3 x 100 mL), sat. NaCl (2 x 200 mL), filtered and evaporated. Ether (25 mL) was added to the residue, and the precipitated product was filtered, washed with ether to give 4-(4'-(aminophenyl)-2-ethylamino)carbonylmethyl -rhod tetramethyl ester, 126 mg (81%) as a dark red solid.

To a solution of 4-(4'-(aminophenyl)-2-ethylamino)carbonylmethyl -rhod tetramethyl ester (100 mg, 0.1 mmol) in dioxane (2 mL), MeOH (2 mL) and H_2O (1 mL) was added 1N KOH to give pH 12.0. The mixture was stirred for 50 h, then the pH was adjusted to 9.0 with 0.1 N HCl. The mixture was evaporated and the residue was purified on a Sephadex LH-20 column (2.6 x 90 cm bed) using H_2O as eluant and lyophilized to give Compound 10, 21 mg (22%) as an orange-red solid.

Example 24: Synthesis of 4-carboxylmethyl 1-4'methoxyrhod-BAPTA, tetrapotassium (Compound 19)

A suspension of (4'-methoxy-2'-nitrophenoxy)-2-chloroethane (11.29 g, 48.7 mmol), methyl (4-hydroxy-3-nitro) phenyl acetate (10.80 g, 51.2 mmol), and K_2CO_3 (13.80 g, 100 mmol) was stirred at 130 °C for 4 h, cooled to room temperature, and poured into ice water (0.8 L), allowed to coagulate 2 days. Precipitate was filtered off, washed with H_2O and dried to give (4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(4''-methoxy-2''-nitrophenoxy)ethane, 15.1 g (76%) as a yellow solid.

The yellow solid, (4'-methoxycarbonylmethyl-2'-nitrophenoxy)-2-(4''-methoxy-2''-nitrophenoxy)ethane (15.0 g, 43.3 mmol) was hydrogenated over 10% Pd/C (2.0 g) in CH_2Cl_2 (250 mL) at 45 psi for 16 h. The mixture was filtered from catalyst through a Celite on a fritted glass filter. The filtrate was evaporated and the residue was treated with ether (200 mL). Precipitated solid was filtered, washed with ether (3 x 25 mL) to give (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane, 11.21 g (75%) as off-white solid.

A mixture of (2'-amino-4'-methoxycarbonylmethylphenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane (8.65 g, 25 mmol), methanol (80 mL), dioxane (80 mL), and 1N KOH (50 mL, 50 mmol) was stirred at 60 °C for 1 h, then overnight at room temperature. The mixture was evaporated and the residue was suspended in H_2O (300 mL). Aqueous 1N HCl was added to pH 4.0. Precipitated product was filtered, washed with H_2O, and dried to give (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane, 6.98 g (84%) as off-white solid.

Diphenyldiazomethane was prepared by vigorously stirring benzophenone hydrazone (5.88 g, 30 mmol) and yellow HgO (17.60 g, 80 mmol) in hexanes (150 mL) for 6 h. The mixture was filtered from inorganics, filtrate was evaporated and the residue was re-dissolved in acetone (40 mL). This solution was added to the solution of (2'-amino-4'-carboxymethyl-1'-phenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane acid (6.64 g, 20 mmol) in acetone (200 mL). The resulting mixture was stirred for 48 h at 35 °C, evaporated and the residue was suspended in CHCl3. To the suspension was added AcOH (4 mL) to decompose the excess reagent and the mixture was stirred for 2 h, then evaporated, and the crude product was purified by flash chromatography on a SiO_2 column (12 x 50 cm bed) using 0.5% MeOH in $CHCl_3$ as eluent to give (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane, 7.81 g (78%) as off-white solid.

A mixture of diamine (2'-amino-4'-diphenylmethoxycarbonylmethylphenoxy)-2-(2''-amino-4''-methoxyphenoxy)ethane (4.62 g, 9.3 mmol), DIEA (52 mL, 300 mmol), methyl bromoacetate (19 mL, 200 mmol), and NaI (0.75 g, 5 mmol) in MeCN (150 mL) was refluxed under stirring for 70 h, cooled to room temperature and evaporated. The residue was dissolved in $CHCl_3$ (400 mL), washed with 1% AcOH (3 x 200 mL), H_2O (200 mL), sat. NaCl (2 x200 mL), filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (12 x 50 cm bed) using gradient 25-40% EtOAc in hexanes as eluent to give 4-diphenylmethoxycarbonylmethyl-4'-methoxy-BAPTA tetramethyl ester, 3.01 g (42%) as a white solid.

To a solution of Vilsmeier reagent made from $POCl_3$ (0.28 mL, 3 mmol) in DMF (2 mL) was added a solution of 4-diphenylmethoxycarbonylmethyl-4'-methoxy-BAPTA tetramethyl ester (762 mg, 1 mmol) in DMF (2 mL). The mixture was stirred for 2 h, then was quickly poured into an ice-sat. K_2CO_3 mixture (50 mL). The mixture was extracted with $CHCl_3$ (7x20 mL), dried over $MgSO_4$ and evaporated. The mixture of products was separated by column chromatography on SiO_2 (4 x 35 cm bed) using gradient 30-45% EtOAc in hexanes to give aldehyde 4-diphenylmethoxycarbonylmethyl-5'-formyl-4'-methoxy-BAPTA tetramethyl ester, 760 mg (96%) as a white solid.

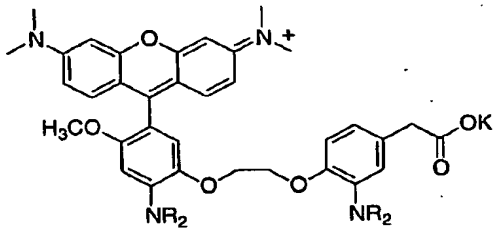
A mixture of aldehyde 4-diphenylmethoxycarbonylmethyl-5'-formyl-4'-methoxy-BAPTA tetramethyl ester (1.58 g, 2.0 mmol), m-dimethylamino phenol (602 mg, 4.4 mmol), and TsOH (50 mg, catalyst) in Et-COOH (20 mL) was stirred at 65 °C for 20 h, then cooled down to room temperature and poured into 3N NaOAc (300 mL). After 1 h coagulation the

precipitated product was filtered, washed with water, and dried to give 4-diphenylmethoxycarbonylmethyl-5'dihydrorhod tetramethyl ester, 2.00 g (99%) as a purple-red solid.

A mixture of compound 4-diphenylmethoxycarbonylmethyl-5'dihydrorhod tetramethyl ester (2.00 g, 1.9 mmol) and powdered chloraniline (0.50 g, 2 mmol) in $CHCl_3$ and MeOH (50 mL of each) was stirred for 4 h, filtered and evaporated. The residue was purified by flash chromatography on a SiO_2 column (6 x 50 cm bed) using gradient 5-7% MeOH in $CHCl_3$/ 0.5% AcOH as eluent to give a crude product, which was re-dissolved in $CHCl_3$, filtered from SiO_2, and evaporated to give 4-(diphenylmethoxycarbonylmethyl)-4'-methoxy-rhod, tetramethyl ester, 480 mg (24%) as a dark purple solid.

To a solution of 4-(diphenylmethoxycarbonylmethyl)-4'-methoxy-rhod, tetramethyl ester (45 mg, 0.04 mmol) in dioxane (1 mL), MeOH (2 mL) and H_2O (2 mL) was added 1N KOH to pH 12.0. The mixture was stirred for 50 h, then pH was adjusted to 9.0 with 0.1 N HCl. The mixture was evaporated and the residue was purified on Sephadex LH-20 column (2.6 x 90 cm bed) using H_2O as eluant and lyophilized to give Compound 19, 12 mg (29%) as a red solid.

Compound 19



The reagents employed in the preceding examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art or whose preparation is described in the examples. It is evident from the above description and results that the subject invention is greatly superior to the presently available methods for staining phosphorylated biomolecules in a biological sample. The subject invention overcomes the shortcomings of the currently used methods by allowing staining as

well as isolation of phosphorylated biomolecules in a simple procedure that has increased sensitivity. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the forgoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A method for staining a phosphorylated biomolecule, said method comprising the steps of:
a) combining said phosphorylated biomolecule with an acid, BAPTA compounds and a gallium salt wherein, said gallium salt comprises trivalent gallium ions, to form a mixture; and,
b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated biomolecule and said BAPTA compound whereby said phosphorylated biomolecule is stained.

2. The method according to Claim 1, wherein said phosphorylated biomolecule, said gallium salt, said acid and said BAPTA compound are combined separately or in combination to form an acidic staining mixture.

3. The method according to Claim 2, wherein said staining mixture has a pH about 3 to about pH 5.

4. The method according to Claim 1, wherein said phosphorylated biomolecules are selected from the group consisting of proteins, peptides, nucleotides and lipids.

5. The method according to Claim 1, wherein said BAPTA compounds comprise one or more labels.

6. The method according to Claim 5, wherein said label is a fluorescent dye, a hapten or an enzyme.

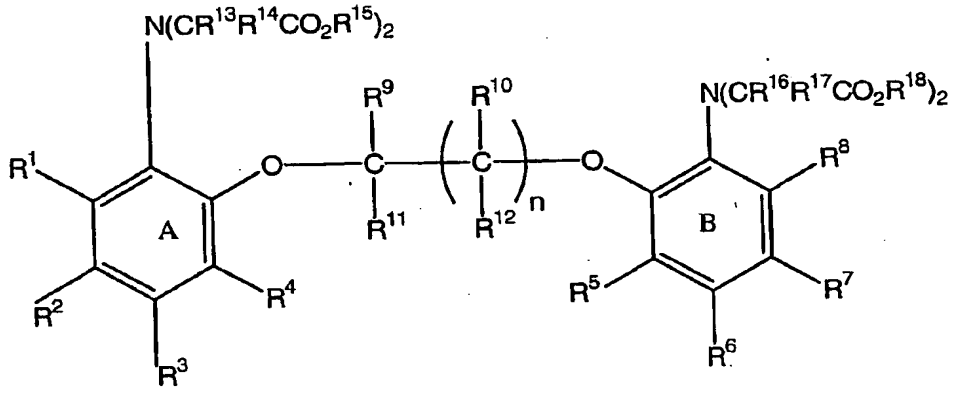
7. The method according to Claim 6, wherein said hapten is a biotin.

8. The method according to Claim 6, wherein said fluorescent dye is selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

9. The method according to any one of Claims 6 or 8, wherein said fluorescent dye is not sulfonated.

10. A method for staining a phosphorylated biomolecule in a sample, said method comprising the steps of:

(a) combining said sample with a staining mixture comprising an acid, a gallium salt wherein, said gallium salt comprises trivalent gallium ions, and labeled BAPTA compounds according to Formula 1;



FORMULA 1

wherein n is 1 or 2;

one of R1-R4 is a label or prolabel wherein said label or prolabel is attached to ring A by a single covalent bond or by a spacer or is fused to ring A wherein said label or prolabel share 2 carbon atoms, alternatively one of R5-R8 is a said label or prolabel;

the remainder of R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, alkoxy, alkyl, aryl, amino, carboxyl, nitro, cyano, thioether, sulfinyl;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents R^9 and R^{10} in combination constitute a 5-membered or 6-membered alicyclic or heterocyclic ring;
R^{13}, R^{14}, R^{16} and R^{17} are independently H or lower alkyl, wherein lower alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R15 and R18 are independently hydrogen or a salt; and,

b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated biomolecule and said labeled BAPTA compound;

c) detecting said labeled BAPTA compound whereby said phosphorylated biomolecule is stained.

11. The method according to Claim 10, wherein said staining mixture is formed prior to or during the step of combining said acid, gallium salt and labeled BAPTA compounds with said sample.

12. The method according to Claim 10, wherein said staining mixture has a pH about 3 to about pH 5.

13. The method according to Claim 10, wherein said gallium salt is gallium chloride.

14. The method according to Claim 10, wherein said phosphorylated biomolecule is selected from the group consisting of proteins, peptides and lipids.

15. The method according to Claim 10, wherein said phosphorylated biomolecule is immobilized on a solid or semi-solid surface or is solubilized in solution.

16. The method according to Claim 15, wherein said solid or semi-solid surface is a gel, a blot, or an array.

17. The method according to Claim 10, wherein said label is a fluorescent dye, an enzyme or a hapten.

18. The method according to Claim 17, wherein said hapten is a biotin.

19. The method according to Claim 17, wherein said labeled BAPTA is fluorescent or fluorogenic and said label is a fluorescent dye selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

20. The method according to Claim 19, wherein said xanthene is an amino/imino xanthene derivative or a dihydroxy xanthene derivative.

21. The method according to Claim 19, wherein said labeled BAPTA comprises a quinazolinone further comprising an adjacent hydroxyl group attached to said BAPTA compound wherein remaining ring substituents are independently selected from fluorine, hydrogen and NCH_2CO_2K.

22. The method according to any one of Claims 19, 20 or 21, wherein said fluorescent dye is not sulfonated.

23. The method according to Claim 19, wherein said labeled BAPTA comprises a fluorescent dye comprising one or two of R1-R4 and is a fluorophore, and R5-R8 is selected from the group consisting of H, NO_2, F, CF_3, lower alkyl, linker-biotin and linker-aniline.

24. The method according to Claim 23, wherein said fluorophore is independently R3, R2 or R3 and R2 together.

25. The method according to Claim 23, wherein said R6 or R7 is a linker-biotin or linker-analine.

26. The method according to Claim 23, wherein said R6 or R5 or both is fluorine.

27. The method according to Claim 23, wherein said R6 is NO_2.

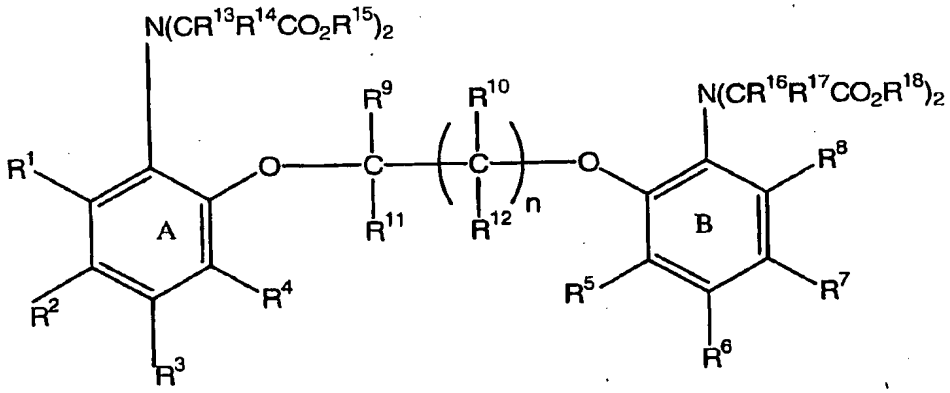
28. The method according to Claim 19, wherein said labeled BAPTA comprises a first fluorophore and a second fluorophore.

29. The method according to Claim 25, wherein said second fluorophore is independently R6, R7 or R6 and R7 together.

30. A method for staining a phosphorylated biomolecule, said method comprising the steps of:

a) immobilizing said phosphorylated biomolecule in a gel, in a blot, or on an array;

b) applying a detection mixture to said gel, blot, or array, wherein said detection mixture comprises an acid, a gallium salt wherein, said gallium salt comprises trivalent gallium ions, and a labeled BAPTA according to Formula 1;



wherein n is 1 or 2;

one of R^1-R^4 is a label or prolabel wherein said label or prolabel is attached to ring A by a single covalent bond or by a spacer or is fused to ring A wherein said label or prolabel share 2 carbon atoms, alternatively one of R5-R8 is a said label or prolabel;

the remainder of R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, alkoxy, alkyl, aryl, amino, carboxyl, nitro, cyano, thioether, sulfinyl;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents R^9 and R^{10} combination constitute a 5-membered or 6-membered alicyclic or heterocyclic ring;
R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R^{15} and R^{18} are independently hydrogen or a salt; and,

b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated biomolecule and said labeled BAPTA compound;

c) detecting said labeled BAPTA compound whereby said phosphorylated biomolecule in a gel, on a blot or on an array is stained.

31. The method according to Claim30, further comprising adding an additional detection reagent to said, gel, blot, or array.

32. The method according to Claim 30, wherein said additional detection reagent is a staining solution specific for total proteins or glycoprotein.

33. The method according to Claim 30, wherein said staining mixture is formed prior to or during the step of combining said acid, gallium salt and labeled BAPTA compounds with said immobilized phosphorylated biomolecules, wherein said staining mixture has a pH about 3 to about pH 5.

34. The method according to Claim 33, wherein said gallium salt is gallium chloride.

35. The method according to Claim 33, wherein said phosphorylated biomolecule is selected from the group consisting of proteins, peptides and lipids.

36. The method according to Claim 30, wherein said array is a protein array or lipid array.

37. The method according to Claim 30, wherein said label is a fluorescent dye, an enzyme or a hapten.

38. The method according to Claim 37, wherein said hapten is a biotin.

39. The method according to Claim 37, wherein said labeled BAPTA is fluorescent or fluorogenic and said label is a fluorescent dye selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

40. The method according to Claim 39, wherein said xanthene is amino/imino xanthene derivative or dihydroxy xanthene derivative.

41. The method according to Claim 39, wherein said labeled BAPTA comprises a quinazolinone further comprising an adjacent hydroxyl group attached to said BAPTA compound wherein remaining ring substituents are independently selected from the group consisting of fluorine, hydrogen and NCH_2CO_2K.

42. The method according to Claim 41, wherein said fluorescent dye is not sulfonated.

43. A method for staining a phosphorylated biomolecule, said method comprising the steps of:

a) immobilizing said phosphorylated biomolecule in a gel, in a blot, or on an array;

c) applying a detection mixture to said gel, blot, or array, wherein said detection mixture comprises an acid, a gallium salt wherein, said gallium salt comprises trivalent gallium ions, and a labeled BAPTA compound wherein said labeled BAPTA compound is selected from the group consisting of compound 1, 3, 4, 5, 9, 10, 11 and 12;

b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated biomolecule and said labeled BAPTA compound;

c) detecting said labeled BAPTA compound whereby said phosphorylated biomolecule in a gel, on a blot or on an array is stained.

44. The method according to Claim 43, further comprising adding an additional detection reagent to said, gel, blot, or array.

45. The method according to Claim 43, wherein said additional detection reagent is a staining solution specific for total proteins or glycoprotein.

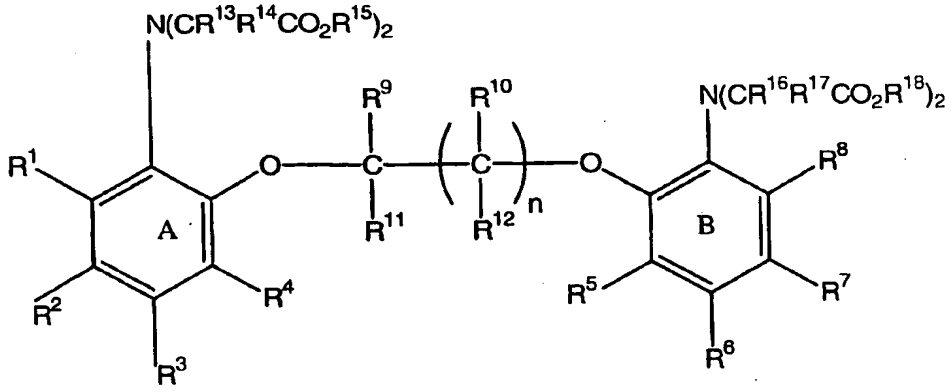
46. The method according to Claim 43, wherein said staining mixture is formed prior to or during the step of combining said acid, gallium salt and labeled BAPTA compounds with said immobilized phosphorylated biomolecules, wherein said staining mixture has a pH about 3 to about pH 5.

47. The method according to Claim 46, wherein said gallium salt is gallium chloride.

48. The method according to Claim 46, wherein said phosphorylated biomolecule is selected from the group consisting of proteins, peptides and lipids.

49. The method according to Claim 48, wherein said array is a protein array or lipid array.

50. A method for isolating soluble phosphorylated peptides or proteins from a complex sample mixture, said method comprising the steps of:
(a) combining said soluble phosphorylated peptides or proteins with an isolation mixture comprising an acid, a gallium salt wherein, said gallium salt comprises trivalent gallium ions, and labeled BAPTA compounds according to Formula 1;



FORMULA 1

wherein n is 1 or 2;

one of R^1-R^4 is a label or prolabel wherein said label or prolabel is attached to ring A by a single covalent bond or by a spacer or is fused to ring A wherein said label or prolabel share 2 carbon atoms, alternatively one of R5-R8 is a said label or prolabel;

the remainder of R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents R^9 and R^{10} in combination constitute a 5-membered or 6-membered alicyclic or heterocyclic ring;
R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R^{15} and R^{18} are independently hydrogen or a salt; and,

b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated proteins or peptides and said labeled BAPTA compound;

c) precipitating said complex from said sample mixture whereby said phosphorylated proteins, peptides or nucleotides are isolated.

51. The method according to Claim 50, wherein said isolation mixture is formed prior to or during the step of combining said acid, gallium salt and labeled BAPTA compounds with said solubilized phosphorylated proteins or peptides wherein said isolation mixture has a pH about pH 3 to about pH 5.

52. The method according to Claim 50, wherein said gallium salt is gallium chloride.

53. The method according to Claim 50, wherein said label is a fluorescent dye, an enzyme or a hapten.

54. The method according to Claims 53, wherein said fluorescent dye is not sulfonated.

55. The method according to Claim 53, wherein said hapten is a biotin.

56. The method according to Claim 54, wherein said labeled BAPTA is fluorescent or fluorogenic and said label is a fluorescent dye selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

57. The method according to Claim 56, wherein said labeled BAPTA comprises a quinazolinone further comprising an adjacent hydroxyl group attached to said BAPTA compound wherein remaining ring substituents are independently selected from the group consisting of fluorine, hydrogen and NCH_2CO_2K.

58. The method according to Claim 57, further comprising the isolation of said phosphorylated peptides or proteins wherein said labeled BAPTA comprises a fluorescent dye and a biotin, said method further comprising the steps of:
a) adding a base to said precipitated phosphorylated proteins or peptides wherein said complex is disassociated into a solution of individual gallium ions, labeled BAPTA compounds and phosphorylated proteins or peptides; and,

b) separating said labeled BAPTA from said phosphorylated proteins or peptides by affinity chromatography, whereby said phosphorylated proteins or peptides are further isolated.

59. The method according to Claim 58, wherein said affinity chromatography comprises a matrix attached to a biotin-binding protein.

60. The method according to Claim 58, wherein said base is barium hydroxide.

61. The method according to Claim 58, wherein said isolated phosphorylated proteins or peptides are analyzed by mass spectrometry.

62. A method for isolating soluble phosphorylated peptides or proteins from a complex sample mixture, said method comprising the steps of:

a) combining said soluble phosphorylated peptides or proteins with an isolation mixture comprising an acid, a gallium salt wherein, said gallium salt comprises trivalent gallium ions, and labeled BAPTA compounds wherein said labeled BAPTA compounds are selected from the group consisting of compound 1, 3, 6, 10 and 13;

b) allowing sufficient time for said gallium ions to form a complex with said phosphorylated proteins or peptides and said labeled BAPTA compound;

c) precipitating said complex from said sample mixture whereby said phosphorylated proteins or peptides are isolated.

63. The method according to Claim 62, wherein said isolation mixture is formed prior to or during the step of combining said acid, gallium salt and labeled BAPTA compounds with said solubilized phosphorylated proteins or peptides wherein said isolation mixture has a pH about pH 3 to about pH 5.

64. The method according to Claim 63, wherein said gallium salt is gallium chloride.

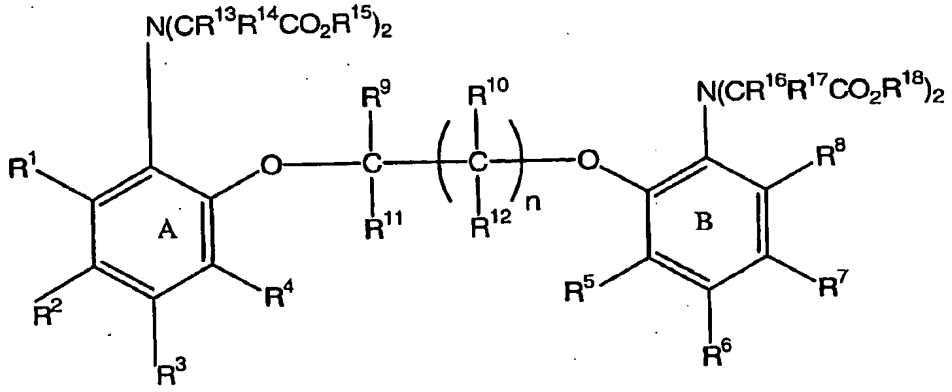
65. The method according to Claim 64, further comprising the isolation of said phosphorylated peptides or proteins wherein said labeled BAPTA is compound 9 or compound 13, said method further comprising the steps of:
a) adding a base to said precipitated phosphorylated proteins or peptides wherein said complex is disassociated into a solution of individual gallium ions, labeled BAPTA compounds and phosphorylated proteins or peptides; and,
b) separating said labeled BAPTA from said phosphorylated proteins or peptides by affinity chromatography, whereby said phosphorylated proteins or peptides are further isolated.

66. The method according to Claim 65, wherein said affinity chromatography comprises a matrix attached to a biotin-binding protein.

67. The method according to Claim 66, wherein said base is barium hydroxide.

68. The method according to Claim 67, wherein said isolated phosphorylated proteins or peptides are analyzed by mass spectrometry.

69. A method for isolating phosphorylated peptides or proteins from a complex sample mixture, said method comprising the steps of:
a) adding a gallium salt to a matrix, wherein said gallium salt comprises trivalent gallium ions and said matrix comprises BAPTA compounds according to formula 1,



FORMULA 1

wherein n is 1 or 2;

R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents in combination constitute R^9 and R^{10} a 5-membered or 6-membered alicyclic or heterocyclic ring;
R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R^{15} and R^{18} are independently hydrogen or a salt; and,

b) equilibrating said matrix with an acidic binding buffer,
b) applying said sample mixture to said matrix wherein said phosphorylated proteins or peptides form a complex with said gallium ions and said BAPTA compounds;
c) eluting said phosphorylated peptides or proteins from said gallium ions with a base whereby said peptides or proteins are isolated.

70. The method according to Claim 69, wherein said BAPTA compounds comprise agarose and said matrix is a column.

71. The method according to Claim70, wherein said matrix comprises compound 13 or compound 14.

72. The method according to Claim 71, wherein said base is barium hydroxide.

73. The method according to Claim 72, wherein said gallium salt is gallium chloride.

74. The method according to Claim 73, wherein said phosphorylated proteins or peptides are analyzed by mass spectrometry.

75. The method according to any one of Claims 58, 65 or 69 further comprising differentially isolating phosphorylated peptides, said method further comprising:

a) adding a nucleophile and a base to a first portion of said isolated phosphorylated peptides;

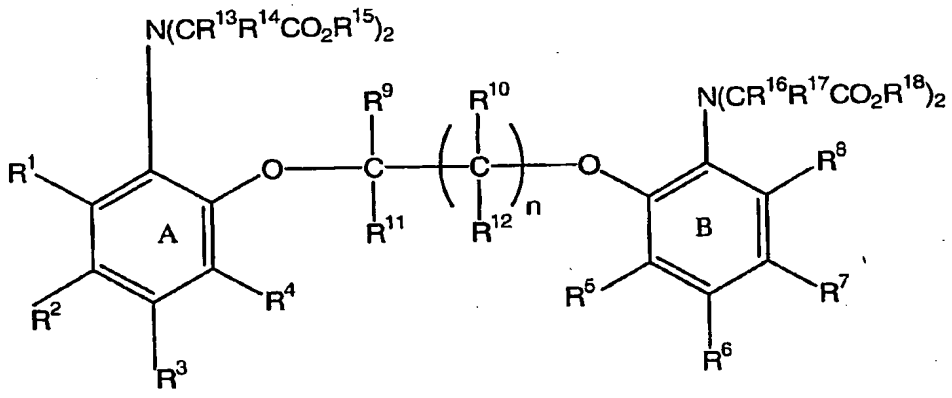
b) adding a base to a second portion of said phosphorylated peptides; and,

c) allowing sufficient time for said base and said nucleophile to derivatize said phosphorylated peptides by β-elimination whereby phosphopeptides comprising phosphoserine, phosphopeptides comprising phosphoserine and phosphothreonine, phosphopeptides comprising phosphotheronine, and phosphopeptides comprising phospotyrosine are differently derivatized.

76. The method according to Claim 75, wherein said phosphorylated peptides are generated by digestion of isolated phosphorylated proteins.

77. A kit for staining phosphorylated biomolecules in a gel, on a blot or on an array, said kit comprising:

A staining mixture comprising an acid, a gallium salt wherein said gallium salt comprises trivalent gallium ions, and a BAPTA compound according to Formula 1;



FORMULA 1

wherein n is 1 or 2;

R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents in combination constitute R^9 and R^{10} a 5-membered or 6-membered alicyclic or heterocyclic ring;

R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R15 and R18 are independently hydrogen or a salt; and,

b) a total protein stain solution; and,

c) molecular weight markers for both phosphorylated proteins and un-phosphorylated proteins.

78. The kit according to Claim 77, wherein said gallium salt is gallium chloride.

79. The kit according to Claim 77, wherein kit optionally does not contain molecular weight markers.

80. The kit according to Claim 77, wherein said kit further comprises an anti-BAPTA antibody.

81. The kit according to Claim 79, wherein said BAPTA compound is a labeled BAPTA compound, wherein one of R^1-R^4 is a label or prolabel wherein said label or prolabel is attached to ring A by a single covalent bond or by a spacer or is fused to ring A wherein said label or prolabel share 2 carbon atoms, alternatively one of R^5-R^8 is a said label or prolabel.

82. The kit according to Claim 81, wherein said label is a fluorescent dye, an enzyme or a hapten.

83. The kit according to Claims 82, wherein said fluorescent dye is not sulfonated.

84. The kit according to Claim 82, wherein said hapten is a biotin.

85. The kit according to Claim 83, wherein said labeled BAPTA is fluorescent or fluorogenic and said label is a fluorescent dye selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

86. The kit according to Claim 85, wherein said labeled BAPTA comprises a quinazolinone further comprising an adjacent hydroxyl group attached to said BAPTA compound wherein remaining ring substituents are independently selected from the group consisting of fluorine, hydrogen and NCH_2CO_2K.

87. A kit for staining phosphorylated biomolecules in a gel, on a blot or on an array, said kit comprising:

a) staining mixture comprising an acid, a gallium salt wherein said gallium salt comprises trivalent gallium ions, and a BAPTA compound selected from the group consisting of compound 1, 3, 4, 5, 9, 10, 11 and 12;

b) a total protein stain solution; and,

c) molecular weight markers for both phosphorylated proteins and un-phosphorylated proteins.

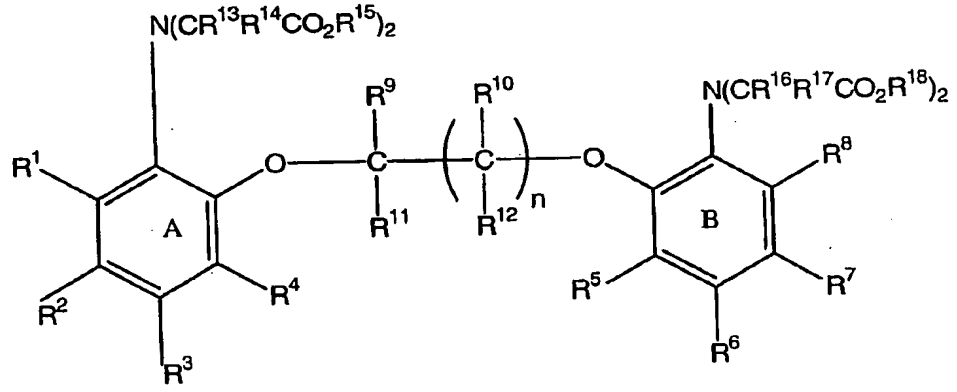
88. The kit according to Claim 87, wherein said gallium salt is gallium chloride.

89. The kit according to Claim 87, wherein said kit further comprises a stain solution specific for glycoproteins.

90. The kit according to Claim 89, wherein kit optionally does not contain molecular weight markers.

91. A kit for preparing a stained phosphorylated biomolecule complex in a sample, said kit comprising:

a) a BAPTA compound according to Formula 1



FORMULA 1

wherein n is 1 or 2;

R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents in combination constitute R^9 and R^{10} a 5-membered or 6-membered alicyclic or heterocyclic ring;

R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R^{15} and R^{18} are independently hydrogen or a salt; and,

b) an acid; and,

c) a gallium salt that comprises trivalent gallium ions.

92. The kit according to Claim 91, wherein said kit further comprises a base.

93. The kit according to Claim 91, wherein said gallium salt is gallium chloride.

94. The kit according to Claim 91, wherein said BAPTA compound is a labeled BAPTA compound, wherein one of R1-R4 is a label or prolabel wherein said label or prolabel is attached to ring A by a single covalent bond or by a spacer or is fused to ring A wherein said label or prolabel share 2 carbon atoms, alternatively one of R5-R8 is a said label or prolabel.

95. The kit according to Claim 94, wherein said label is a fluorescent dye, an enzyme or a hapten.

96. The kit according to Claim 95, wherein said fluorescent dye is not sulfonated.

97. The kit according to Claim 95, wherein said hapten is a biotin.

98. The kit according to Claim 96, wherein said labeled BAPTA is fluorescent or fluorogenic and said label is a fluorescent dye selected from the group consisting of a benzofuran, a quinoline, quinazolinone, a xanthene, an indole, a benzazole and a borapolyazaindacene.

99. The kit according to Claim 98, wherein said labeled BAPTA comprises a quinazolinone further comprising an adjacent hydroxyl group attached to said BAPTA compound wherein remaining ring substituents are independently selected from the group consisting of fluorine, hydrogen and NCH_2CO_2K.

100. A kit for preparing a stained phosphorylated biomolecule complex in a sample, said kit comprising:

a) a BAPTA compound selected from the group consisting of compound 1, 3, 6, 10 and 13;

b) an acid;

c) a gallium salt which comprises trivalent gallium ions; and,

d) a base.

101. The kit according to Claim 100, wherein said gallium salt is gallium chloride.

102. A kit for isolating phosphorylated peptides or proteins from a sample with a BAPTA matrix, said kit comprising;

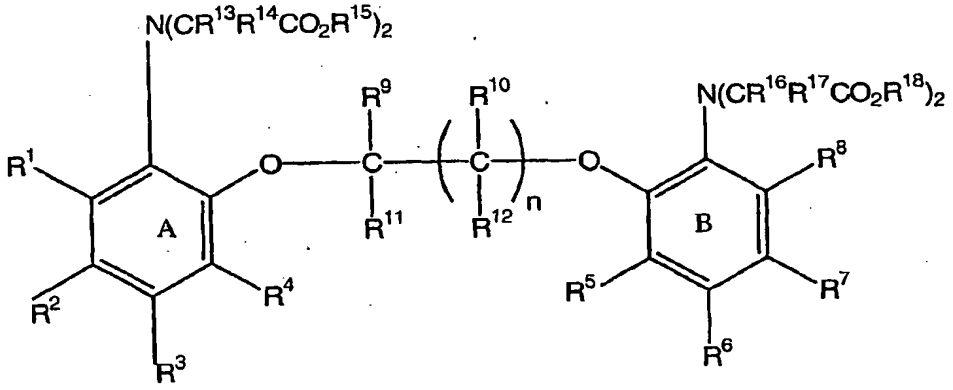
a) a gallium salt wherein said gallium salt comprises trivalent gallium ions;

b) a wash buffer;

c) an acidic binding buffer;

d) a basic elution buffer; and,

a) an agarose column comprising a BAPATA compound according to Formula 1;



FORMULA 1

wherein n is 1 or 2;

R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents in combination constitute R^9 and R^{10} a 5-membered or 6-membered alicyclic or heterocyclic ring;

R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

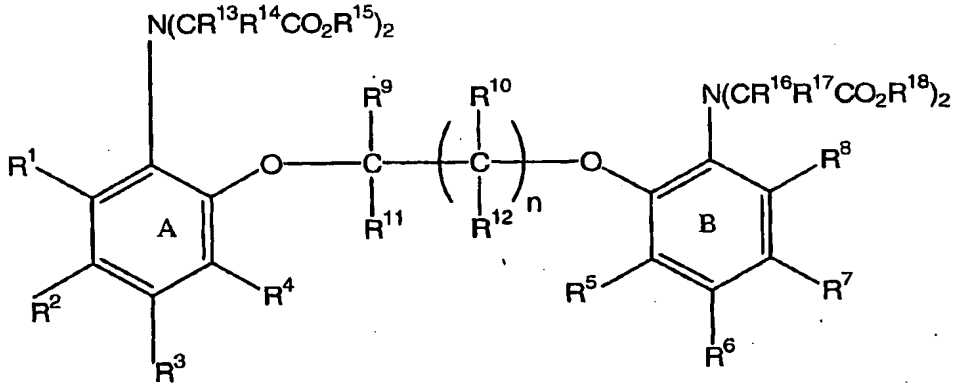
R15 and R18 are independently hydrogen or a salt.

103. The kit according to Claim 102, wherein said gallium salt is sodium chloride.

104. The kit according to Claim 103, wherein said basic buffer comprises barium hydroxide.

105. A kit for isolating phosphorylated peptides or proteins from a sample with a BAPTA matrix, said kit comprising;
a) a gallium salt wherein said gallium salt comprises trivalent gallium ions;
b) a wash buffer;
c) an acidic binding buffer;
d) a basic elution buffer; and,
a) an agarose column comprising compound XX (BAPTA-agarose)

106. An immobilized affinity chromatography column comprising a BAPTA compound according to Formula 1;



FORMULA 1

wherein n is 1 or 2;

R^1-R^8 are selected independently from the group consisting of hydrogen, halogen, hydroxyl, alicyclic, heteroalicyclic, alkyl, aryl, amino, aldehyde, carboxyl, nitro, cyano;

R^9, R^{10}, R^{11} and R^{12}, are independently selected from the group consisting of hydrogen and lower alkyl, or adjacent substituents in combination constitute R^9 and R^{10} a 5-membered or 6-membered alicyclic or heterocyclic ring;
R^{13}, R^{14}, R^{16} and R^{17} are independently H, lower alkyl or alkyl, wherein alkyl is unsubstituted or substituted by carboxyl or alkoxy;

R15 and R18 are independently hydrogen or a salt.

107. The column according to Claim 106 wherein said BAPTA compound comprises agarose.

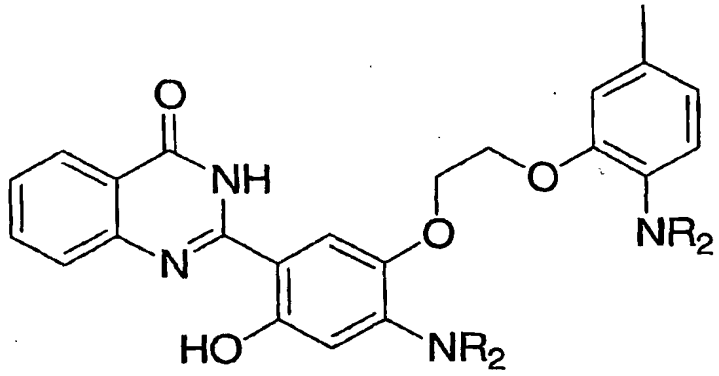
108. An immobilized affinity chromatography column comprising compound 13 or compound 14.

109. A staining mixture comprising an acid, a gallium salt and a BAPTA compound according to Formula 1:

111. A staining mixture comprising an acid, a gallium salt and a BAPTA compound selected from the group consisting of compound 1, 3, 4, 5, 8, 9, 10, 11 and 12

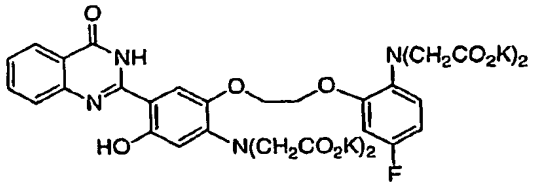
112. An isolation mixture comprising an acid, a gallium salt and a BAPTA compound selected from the group consisting of compound 1, 3, 10 and 13.

113. A labeled BAPTA compound comprising compound 6:



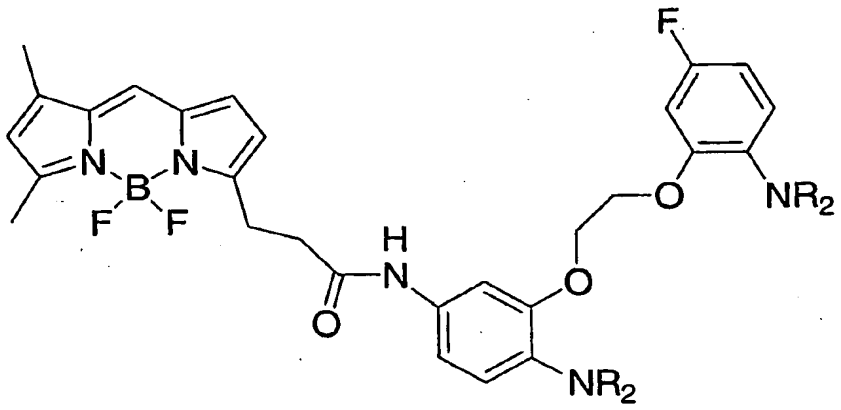
wherein R is CH_2CO_2K and said BAPTA compound comprises a fluorescent label of quinazolinone with an adjacent hydroxyl group.

114. A labeled BAPTA compound comprising compound 7:



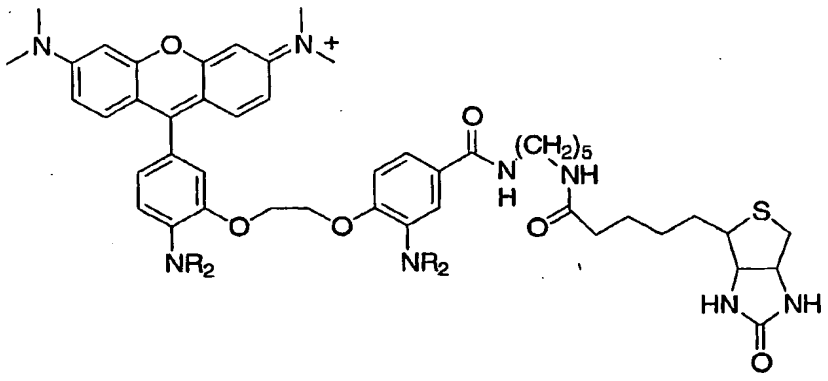
wherein said BAPTA compound comprises a fluorescent dye of quinazolinone with an adjacent hydroxyl group.

115. A BAPTA compound comprising compound 8:



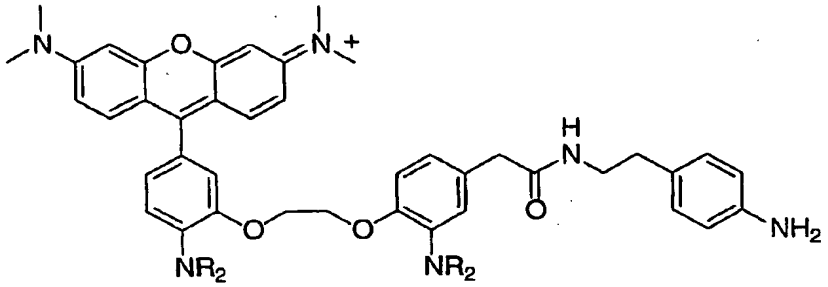
wherein R is CH_2CO_2Na and said BAPTA compound comprises a fluorescent dye wherein said dye is borapolyazaindacene.

116. A labeled BAPTA compound comprising compound 9:



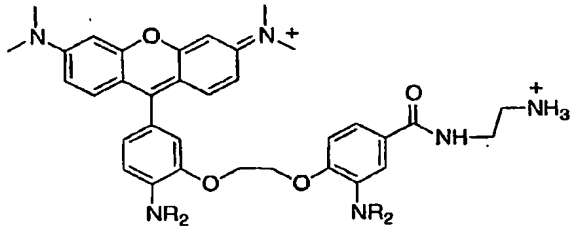
wherein R is CH_2CO_2K and said BAPTA compound comprises a fluorescent dye and a biotin label wherein said fluorescent dye is a xanthene and said biotin is attached by a linker to said BAPTA compound.

117. A labeled BAPTA compound comprising compound 10:

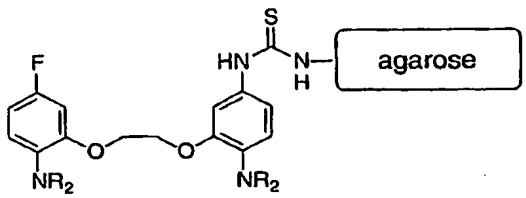


wherein R is CH_2CO_2K and said BAPTA compound comprises a fluorescent dye and an aniline substituent wherein said fluorescent dye is xanthene and said aniline is attached by a linker to said BAPTA compound.

118. A labeled BAPTA compound comprising compound 11:



119. A compound according to compound 14:



NOVEL BAPTA COMPOSITIONS AND METHODS FOR STAINING PHOSPHOPHORYLATED BIOMOLECULES

ABSTRACT

The present invention relates to phospho-sensing complexes that find use in staining, detecting and isolating phosphorylated biomolecules. A staining mixture is provide that comprises a BAPTA compound and a metal ion wherein the metal ion interacts with a phosphate group forming a bridge between the metal chelating compound and a phosphorylated biomolecule resulting in a tertiary complex. The methods involve combining phosphorylated biomolecules with BAPTA compounds, an acid and a gallium salt to form an acidic mixture, wherein trivalent gallium ions bind with high affinity to the BAPTA compounds and phosphorylated biomolecules. The resulting tertiary complex can be visualized when the BAPTA compound optionally comprises a detectable label. The staining mixture of the present invention finds use in staining and detecting immobilized and solubilized phosphorylated biomolecules, isolation of phosphorylated biomolecules from a complex mixture and aiding in proteomic analysis.

Figure 1A

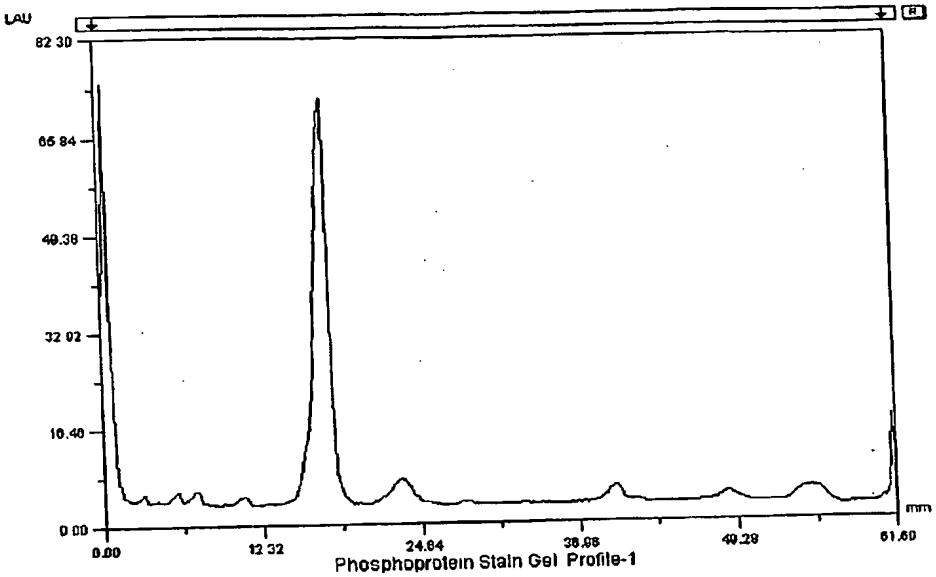


Figure 1B

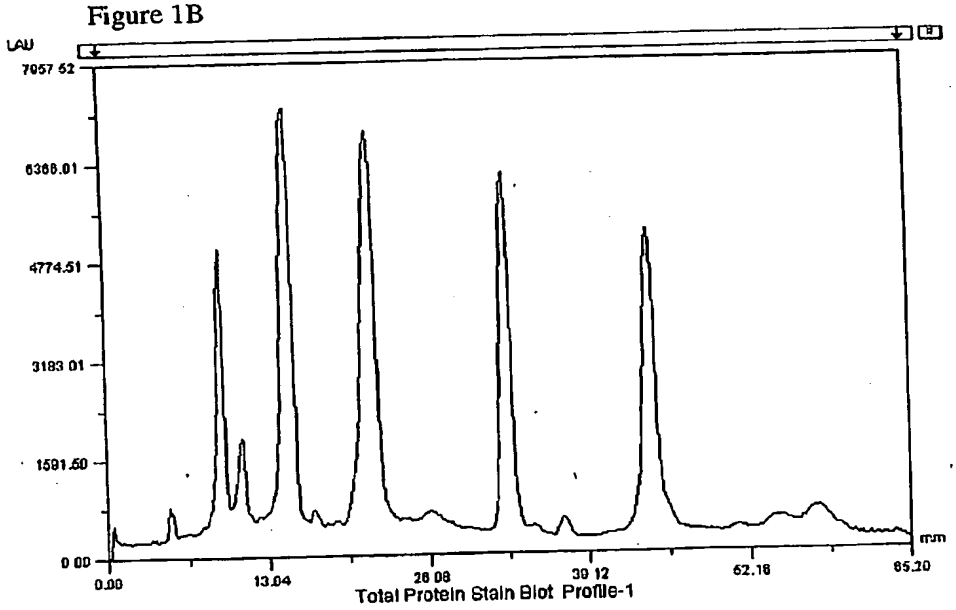


Figure 2A

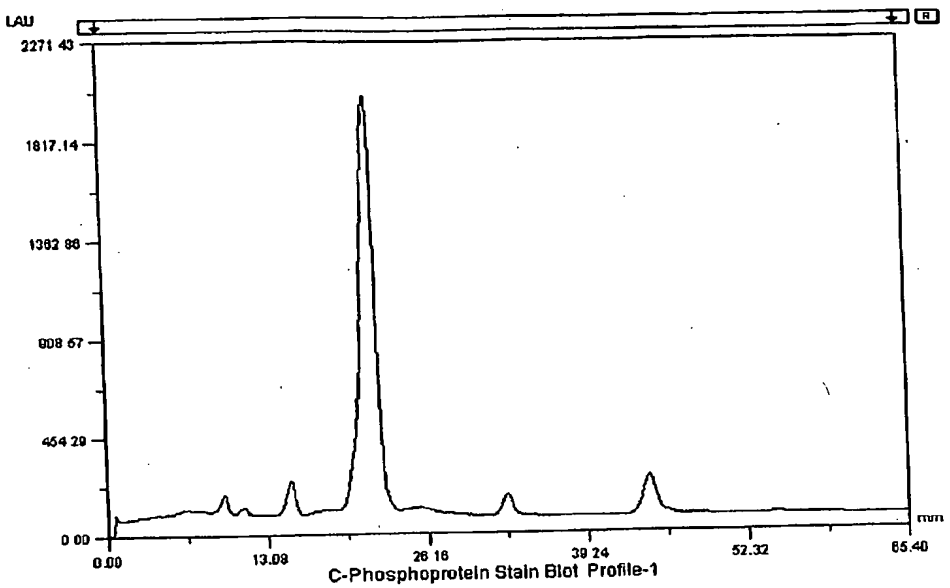


Figure 2B

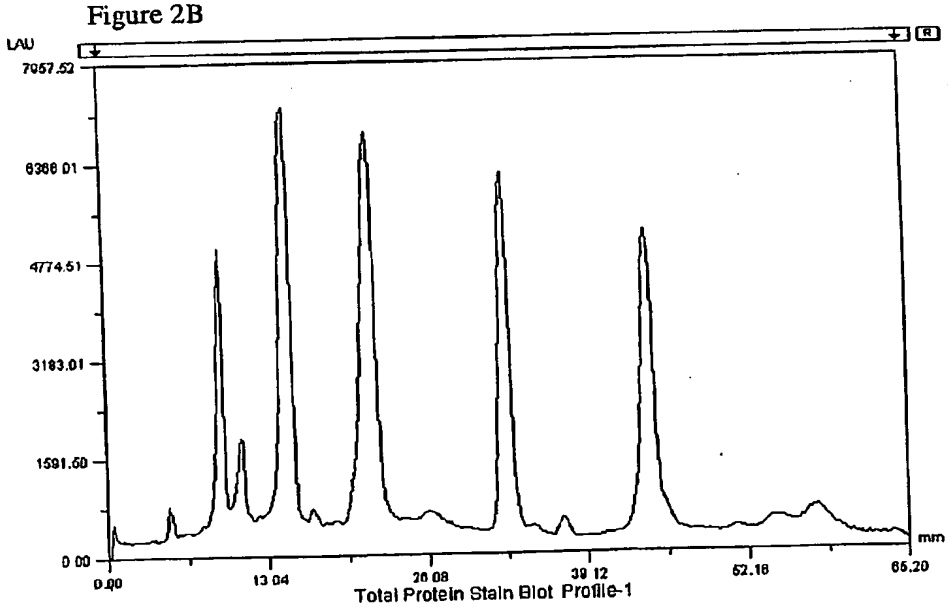


Figure 3A

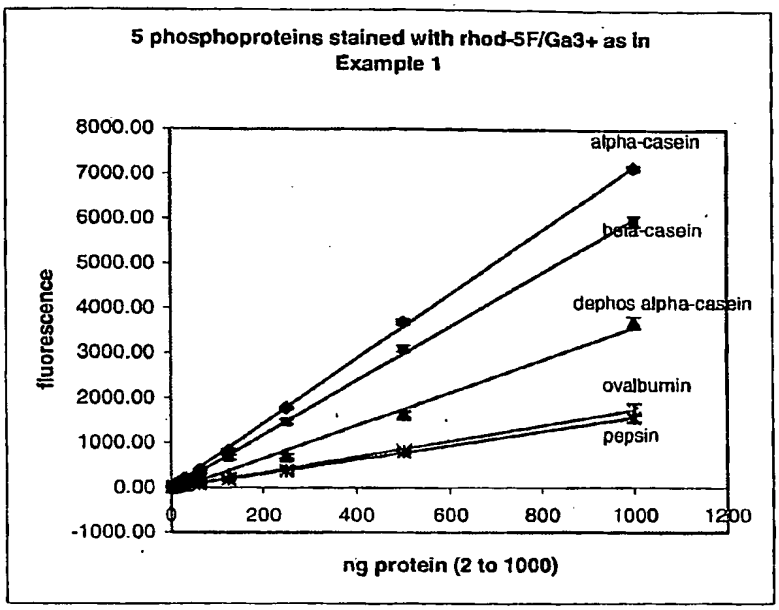


Figure 3B

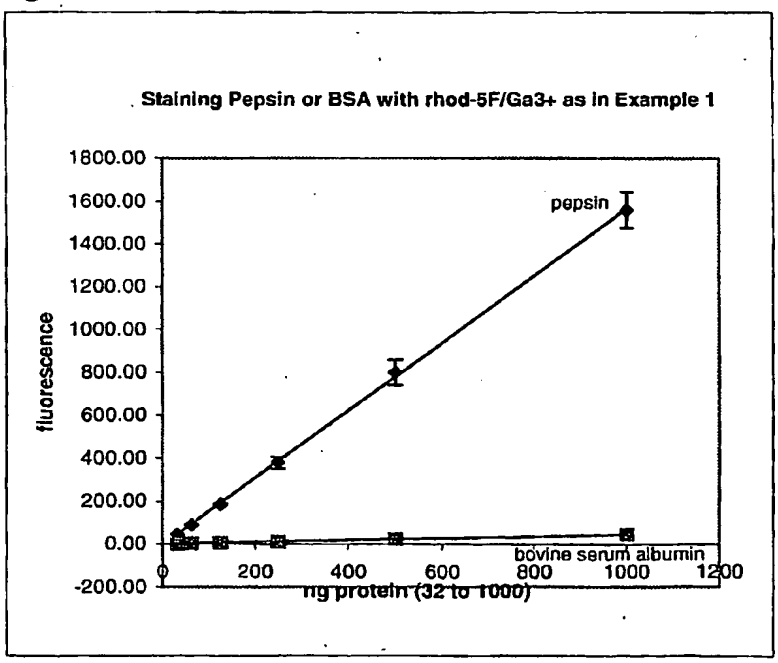


Figure 4

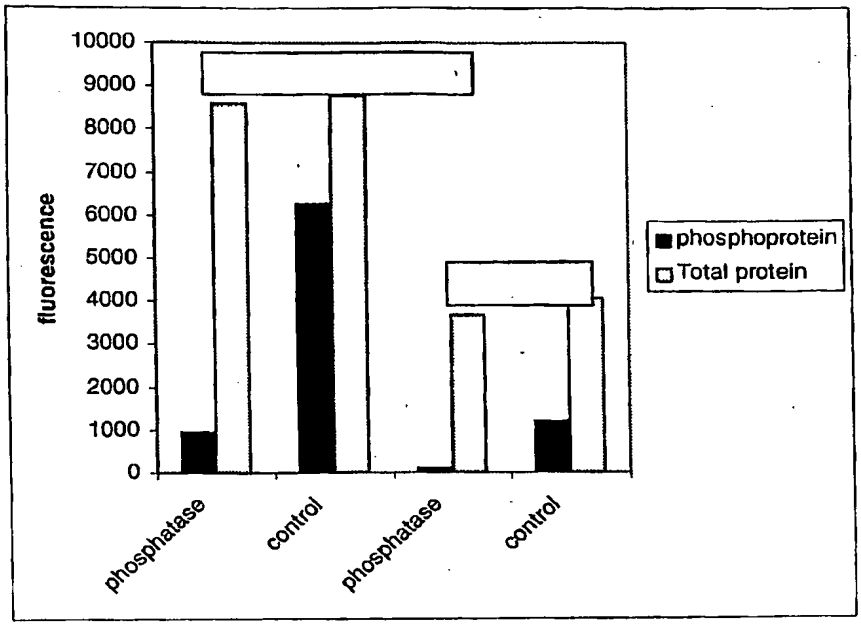


Figure 5

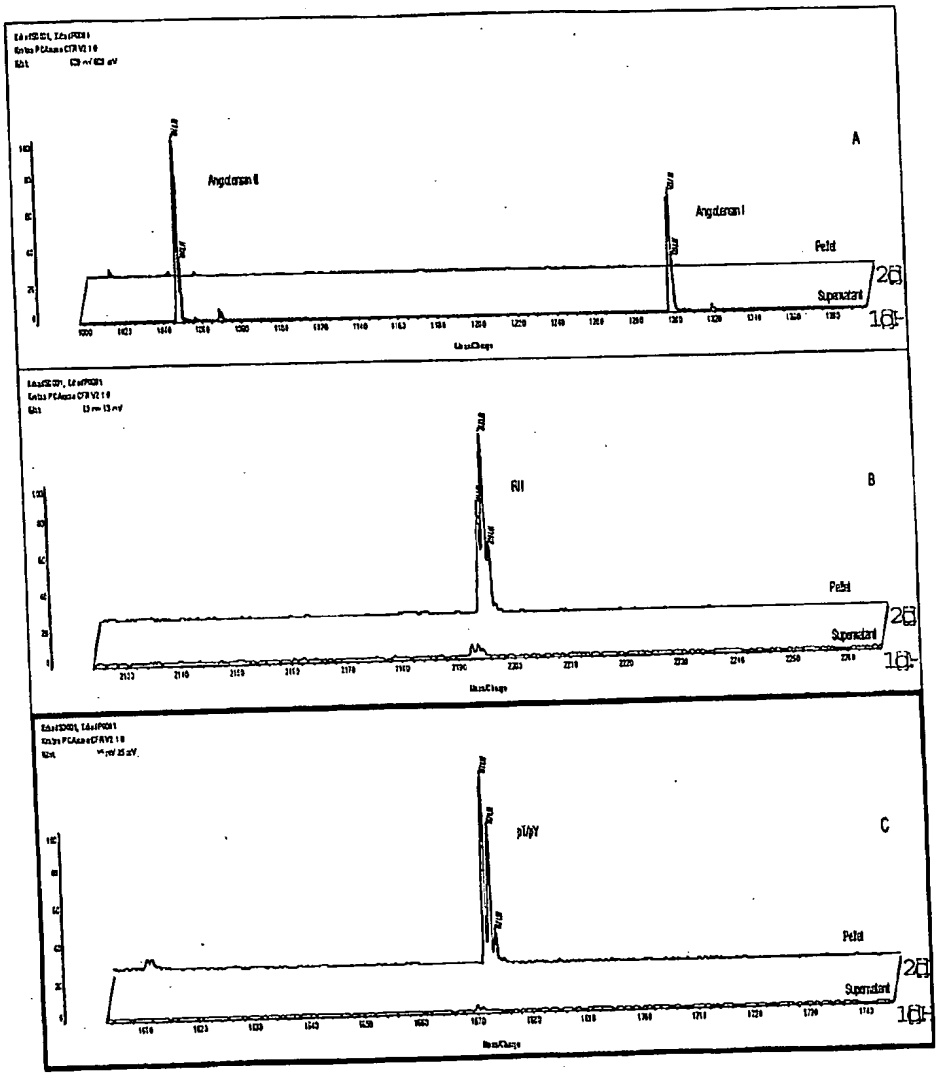


Figure 6